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THE MECHANISM OF STIMULATION OF PHOSPHATIDYLCHOLINE BIOSYNTHESIS BY 12-*O*-TETRADECANOYLPHORBOL-13-ACETATE IN HELA CELLS

BY

© AMANDIP KAUR UTAL

A thesis

submitted to the Faculty of Graduate Studies and Research in

partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

DEPARTMENT OF BIOCHEMISTRY

Edmonton, Alberta

SPRING 1992

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ISBN 0-315-73226-1

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DEGREE	:	Doctor of Philosophy
YEAR THIS DEGREE GRANTED	:	Spring 1992

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DATED: December 23 ,1991

Those Himalays Of The Mind
Are Not So Easily Possessed:

There's More Than Precipice And Storm
Between You And Your Everest.

Cecil Day-Lewis
(1904-1972)

UNIVERSITY OF ALBERTA

FACULTY OF GRADUATE STUDIES AND RESEARCH

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DEDICATION

To my Mentor Dennis E. Vance; my Father, Sukhdev S. Utal; my Brother, Dalbir S. Utal, and most of all to my Mother, Gurdev K. Utal who is my beacon and my inspiration.

To all of you, Thank You Now and Always.

ABSTRACT

The mechanism by which phosphatidylcholine biosynthesis is stimulated by treatment of HeLa cells with 12-*O*-tetradecanoylphorbol 13-acetate (TPA) was investigated. TPA caused a three-fold increase in particulate CTP:phosphocholine cytidylyltransferase activity in HeLa cells which correlated with decreased cytidylyltransferase activity in the cytosol. Similarly, an increase in membrane-associated cytidylyltransferase after TPA treatment of HeLa cells was demonstrated by immunoblotting. Immunoprecipitation studies suggested that TPA had no effect on the state of phosphorylation of cytidylyltransferase. The enhanced binding of cytidylyltransferase to membranes could not be explained by changes in the levels of fatty acid or phosphatidylcholine. Diacylglycerol was considered a possible signal since an increase in the level of diacylglycerol in membranes has been previously shown to enhance binding of cytidylyltransferase to membranes. The level of diacylglycerol in TPA-treated HeLa cells was measured and found to be increased approximately 2-fold (2.29 to 4.02 nmol/mg protein) after 1 h of TPA treatment. In time course experiments, a temporal relationship was shown in which production of diacylglycerol appeared to signal translocation of cytidylyltransferase to membranes followed by a stimulation of phosphatidylcholine biosynthesis. In another approach to test the potential for diacylglycerol to stimulate cytidylyltransferase binding to membranes, the protein kinase C activity of HeLa cells was downregulated and the cells were incubated with the cell permeable diacylglycerol, dioctanoylglycerol. This treatment resulted in almost a 2-fold increase in both cytidylyltransferase activity on membranes and the rate of phosphatidylcholine biosynthesis. Time course experiments with

dioctanoylglycerol revealed a strong positive correlation (r^2=0.89) between the amount of particulate cytidylyltransferase activity and the rate of phosphatidylcholine biosynthesis. In addition, dioctanoylglycerol was shown to be incorporated into PC by radiolabeling and gas liquid chromatographic techniques. It was concluded that a TPA-mediated increase in diacylglycerol concentration stimulates phosphatidylcholine biosynthesis by causing a translocation of CT from cytosol to membranes.

Other possible mechanisms such as enzyme aggregation and involvement of the cytoskeleton in TPA-elicited CT translocation were eliminated. Cytochalasin E, an anti-microfilament agent enhanced TPA-stimulated PC biosynthesis via enhanced CT translocation. Evidence suggested that calcium mediated the cytochalasin E-enhanced CT association with membranes via 'calcium bridges'.

ACKNOWLEDGEMENTS

The most satisfying aspect of the culmination of any piece of work is when one can acknowledge those who were a part of it.

Therefore, I would like to thank my supervisor, Dr. Dennis E. Vance for his encouragement and support, and for always being there whenever I needed to discuss any aspects of my project. I wish to thank him for his standards of excellence and fairplay, standards to which I shall always strive to aspire to.

Members of my committee were always supportive and helpful. Dr. David N. Brindley, in particular, had very helpful hints about technical aspects of my work and willingly spared time and effort to offer advice. In addition, he was instrumental in mobilizing many of us to go skiing.

Dr. Carol Cass offered me encouragement and advice through some difficult times, in addition to the time she spent reading my thesis. Dr. Wolfgang Schneider was always there to offer a word or two of advice about work and other things. I also wish to thank Dr. Robert Campenot for reading through my thesis.

Members of the Lipid and Lipoprotein Research Group (a.k.a., the Grease Pit!) were invaluable with their advice and technical expertise. In particular Dr. Jean Vance offered critical insight into some experiments and was invaluable in her criticisms of our manuscripts - all this in addition to being a truly gracious hostess. Drs. Bob Ryan and Shinji Yokoyama helped with their technical expertise and advice in the lipid field, not forgetting Dr. Gary Lopaschuck, who struck terror (well, apprehension anyway) into the hearts of graduate students at Journal Club Seminars.

The members of my lab - Sandi, in particular, I wish to thank for supervising the lab as only she is capable of. It was a pleasure to work in such a well-oiled lab. I wish to thank Dr. Haris Jamil for listening to my complaints patiently and helping me find my feet when I first joined the lab. Former students Zemin Yao and Jasbinder Sanghera were also of great help. Dr. Grant Hatch was helpful with his expertise in lipid biochemistry and was always game for an 'amicable' discussion or two about the Oilers versus the Winterpeg Jets! I wish to thank Dr. Zheng Cui for his advice and dispelling my naïvety about many things. Darren Fast, our very own computer whiz was very

patient and helpful especially when I got mad at the computer! Drs. Tomoko Mogami and Lilian Tijburg were very helpful, both as scientists and as friends.

Dr. David Severson (University of Calgary) offered invaluable advice and insight during discussions at conferences.

Several individuals who are presently visiting the lab, Drs. George Helmpkamp (from the University of Kansas and without whose enthusiatic support section 1.9 of this thesis might not have been written), Howard Parsons (from the U. of Cowtown, no less!), Henkjan Verkade and François Tercé were wonderful in making the lab a cheerful place.

From the Brindley Lab: Dr. Ashley Martin was always forthcoming with good advice, *Asante Sana* Dr. Zaharali Jamal for your kindness and 'conversations' in Swahili!

I wish to thank the Dept. of Biochemistry for generous financial assistance, and for the advice and help of Dr. Bill Bridger, the Head, not forgetting Dr. John Colter who always made it a point to inquire about my progress even after he had retired.

On a more personal note, there are many people in Edmonton (too numerous to list here) who showed me great kindness during my years here, in particular Laura DiLaurenzio, Rosemary Garner and Dr. Ashok Taneja who saw me through some truly difficult times. The Kahlons always saw that I had a place to go to during Christmas and Thanksgiving. I would also like to thank Neeta and Harkirat Sidhu for their friendship during my final year.

And finally, thank you Edmonton, for having me. You were good to me and taught me some very important lessons - like appreciating cold winters and good hockey!

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LIST OF ABBREVIATIONS

A	ampere
ACS	aqueous counting scintillant
AdoHcy	*S*-Adenosyl-L-homocysteine
AdoMet	*S*-Adenosyl-L-methionine
AT	acylCoA:lysophosphatidylcholine acyltransferase
ATP	adenosine triphosphate
BCA	bicinchoninic acid
BF_3	boron trifluoride
BSA	bovine serum albumin
$CaCl_2$	calcium chloride
cAMP	adenosine 3′, 5′-monophosphate
cDNA	complementary DNA
CDP	cytidine diphosphate
CE	cytochalasin E
CHO	Chinese Hamster Ovary
Ci	Curie
CK	choline kinase
CoA	coenzyme A
CPT	CDP-choline:1,2-diglyceride phosphocholine cytidylyltransferase
CT	CTP:phosphocholine cytidylyltransferase
CTP	cytidine triphosphate
DEGS	diethyleneglycol succinate
DETAPAC	diethylenetriaminepentaacetic acid
DG	diacylglycerol
DHAP	dihydroxyacetone phosphate
DiC_8	1,2-*sn*-dioctanoylglycerol
DiC_8-PC	1,2-*sn*-dioctanoylphosphatidylcholine
DMEM	Dulbecco's modified Eagle's medium
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
dpm	disintegrations per minute
DTT	dithiothreitol
DZA	deazaadenosine
ECL	enhanced chemiluminescence
EDTA	ethylenediaminetetraacetate
EGTA	ethyleneglycol bis-(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid
ER	endoplasmic reticulum
FBS	fetal bovine serum
Fig.	figure
g	gram
G418	genetamycin sulfate
GLC	gas liquid chromatography
GK	glycerol kinase
GPO	glycerol-3-phosphate oxidase
h	hour
H_2O_2	hydrogen peroxide
H-7	1-(5-isoquinolinesulfonyl)-2-methylpiperazine
HBS	hepes buffered saline
HCl	hydrochloric acid

HRP	horseradish peroxidase
HEPES	N-2-hydroxyethylpiperazine-N´-2-ethanesulfonate
HPLC	high performance liquid chromatography
IgG	immunoglobulin G
kDa	kilodaltons
KH_2PO_4	potassium dihydrogen phosphate
KLH	keyhole limpet hemocyanin
Km	Michealis constant of an enzyme reaction
l	litre
LDH	lactate dehydrogenase
LPC	lysophosphatidylcholine
LPL	lipoprotein lipase
LTR	long terminal repeat
LysoPA	lysophosphatidic acid
m	metre
M	molar
MDCK	Madin-Darby Canine Kidney
$MgCl_2$	magnesium chloride
min	minute
Mo-MuLV	mouse murine leukemia virus
MW	molecular weight
mRNA	messenger RNA
NaCl	sodium chloride
NADH	nicotinamide adenine dinucleotide
NaF	sodium fluoride
NBD	4-nitro-2-oxa-1,3-diazole
NMR	nuclear magnetic resonance
OaG	1-oleoyl-2-acetylglycerol
OaPA	1-oleoyl-2-acetylphosphatidic acid
OG	octyl-β-D-glucoside
PA	phosphatidic acid
PAF	platelet activating factor
PAGE	polyacrylamide gel electrophoresis
PAP	phosphatidate phosphohydrolase
PBS	phosphate buffered saline
PC	phosphatidylcholine
PE	phosphatidylethanolamine
PEMT	phosphatidylethanolamine-*N*-methyltransferase
PI	phosphatidylinositol
PIP_2	phosphatidylinositol-4,5-bisphosphate
PKC	protein kinase C
Plase A	phospholipase A
Plase C	phospholipase C
Plase D	phospholipase D
PDME	phosphatidyl-*N*-dimethylethanolamine
PMME	phosphatidyl-*N*-monomethylethanolamine
PMSF	phenylmethylsulfonyl fluoride
PS	phosphatidylserine
psi	pounds per square inch
RNA	ribonucleic acid
rpm	revolutions per minute
S.D.	standard deviation
SDS	sodium dodecyl sulfate

SV 40	simian virus 40
TBS	tris buffered saline
TLC	thin layer chromatography
TPA	12-*O*-tetradecanoylphorbol-13-acetate
Tris	tris (hydroxymethyl) aminomethane
Triton X-100	octyl phenoxy polyethoxyethanol
UV	ultraviolet
v	volume
V	volt
Vmax	maximum velocity of an enzyme reaction
w	weight

CHAPTER 1 INTRODUCTION

The literature on the regulation of phosphatidylcholine (PC) biosynthesis is vast and contains several excellent reviews, including those by Vance and Choy (1979); Pelech and Vance (1984a); Pelech and Vance (1984b); Vance and Pelech (1984); Pelech, Audubert and Vance (1985); Vance and Pelech (1985); Pelech and Vance (1989); Vance (1990); Kent (1990); Kent *et al.*, (1991). These reviews deal in considerable detail with the regulation of PC biosynthesis, in particular the mechanisms of regulation of activation of CTP:phosphocholine cytidylyltransferase (CT), the rate-limiting enzyme in PC biosynthesis. The aim of section 1.1, therefore, is not to repeat the material in these reviews but to provide a brief overview of the general pathways of PC biosynthesis, and update relevant literature.

PC is quantitatively the most important phospholipid constituent of mammalian cell membranes but is absent from most prokaryotic cells. The lipid has long been known to be important for a variety of reasons; it is a major structural constituent of cell membranes as well as lipoproteins and bile. In addition, PC is a critical component of lung surfactant, and also serves as a donor of the fatty acyl moiety in the synthesis of cholesterol ester in plasma in a reaction catalyzed by lecithin-cholesterol acyltransferase. The arachidonic acid for the synthesis of the mediators of inflammation is derived from PC. Platelet activating factor (PAF) is a particular molecular species of PC (1-alkyl-2-acetyl-PC) and is a potent biological agent (active at 10^{-10} M) causing platelet aggregation as well as being a potent vasodepressor compound. It is generally understood that PC is essential to life as we know it, primarily because there are no known diseases related to PC metabolism.

The aforementioned examples of the importance of PC, however, are the more traditional concepts regarding the role of PC, in particular that of maintaining membrane integrity. Research on PC has undergone a renaissance, particularly in the last 5 years due to the exciting discovery that PC can contribute to signal transduction pathways by providing second messengers (Besterman *et al.*, 1986; Daniel *et al.*, 1986; review by Exton, 1990).

1.1. PATHWAYS AND ENZYMES OF PHOSPHATIDYLCHOLINE BIOSYNTHESIS

There are three major pathways for PC biosynthesis, as shown in Fig. 1 all of which do not contribute equally towards the production of PC in cells. The liver has traditionally been the organ of choice for studying PC metabolism mainly because this organ produces PC not only for membrane biogenesis but also for export in lipoproteins and bile. Pulmonary PC metabolism has also been the target of intense study particularly after PC was identified as a critical component of lung surfactant (Pattle and Thomas, 1961; Klaus *et al.*, 1961). In recent years, however, the study of PC metabolism has shifted somewhat to other cell lines. This shift in focus is in most part due to the discovery that PC can participate in signal transduction.

1.1.1. *The CDP-choline Pathway*

The CDP-choline pathway is also known as the Kennedy Pathway after Eugene P. Kennedy whose pioneering work described most of the enzyme reactions in the biosynthesis of PC via this route (Kennedy and Weiss, 1955; Kennedy and Weiss, 1956; Smith *et al.*, 1958) and is shown in Fig. 2. This is the major route for PC biosynthesis. 80% of hepatic PC is made from choline via CDP-choline (Sundler and Akesson, 1975).

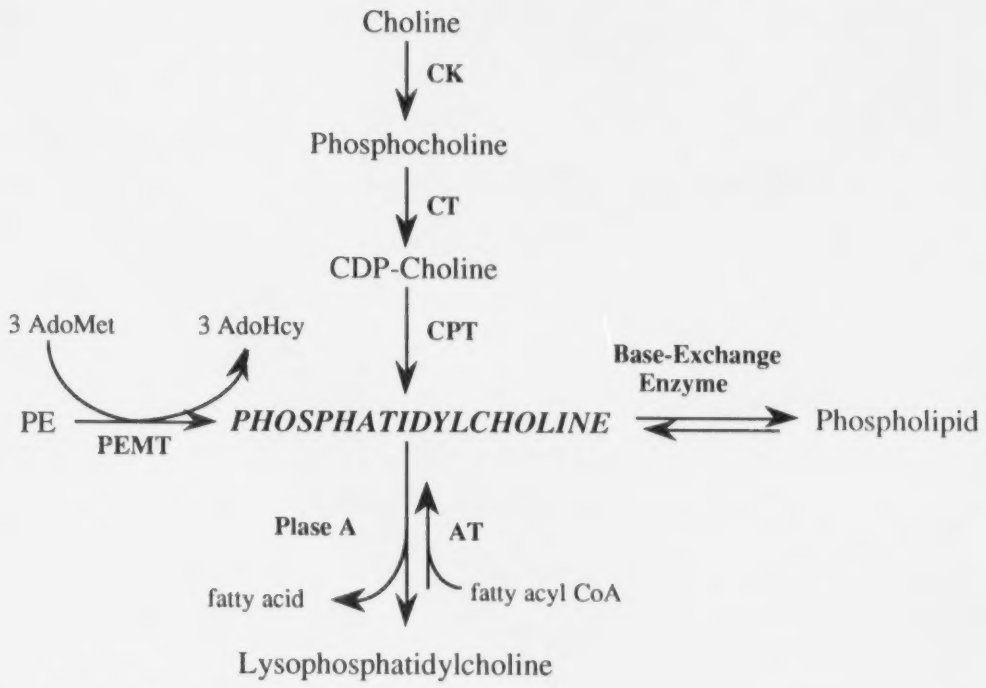


Fig. 1. PC Biosynthetic Pathways. Abbreviations used are: **AT**, acylCoA:lysophosphatidylcholine acyltransferase; AdoHcy, *S*-adenosylhomocysteine; AdoMet, *S*-adenosylmethionine; **CK**, choline kinase; **CT**, CTP:phosphocholine cytidylyltransferase; **CPT**, CDP-choline:1,2-diglyceride phosphocholinetransferase; PE, phosphatidylethanolamine; **PEMT**, phosphatidylethanolamine *N*-methyltransferase; **Plase A**, phospholipase A.

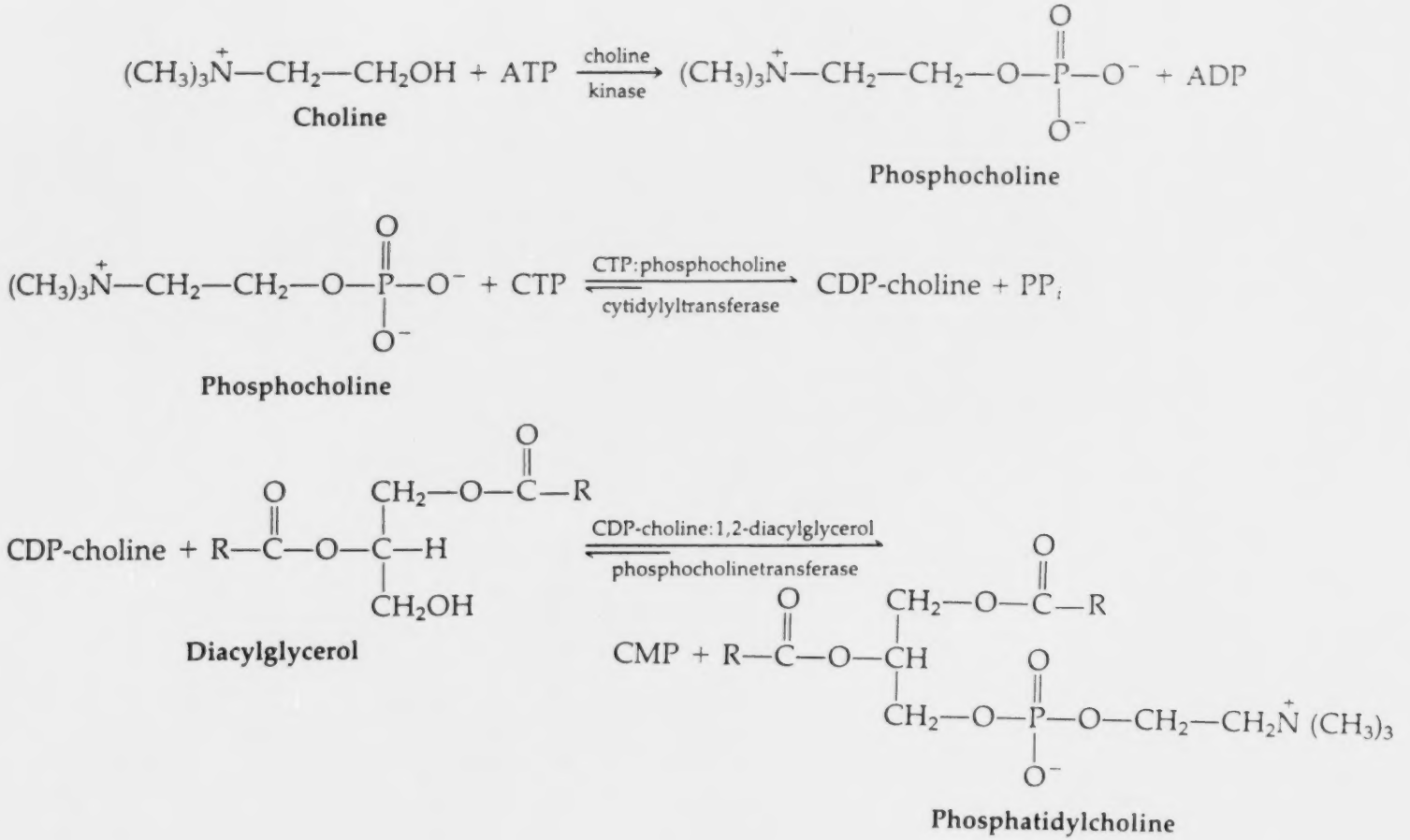


Fig. 2. The CDP-Choline (Kennedy) Pathway for *de novo* PC Biosynthesis (from *Biochemistry of Lipids and Membranes*, Eds., Vance, D.E. and Vance, J.E., The Benjamin/Cummings Publishing Company, Inc., Menlo Park, California, 1985), with permission.

a) Choline transport and phosphorylation - Choline is a dietary requirement that is transported into cells by a carrier-mediated process. The kinetics of choline transport into animal cells and cholinergic nerve tissues are reviewed by Ishidate (1989). Cholinergic synaptosomes transport choline via a high affinity transporter (Km < 5 μM) which is tightly coupled to acetylcholine synthesis, whereas most animal cells as well as cholinergic nerve tissues transport choline via a low affinity transporter with a Km for choline > 20 μM. The intracellular degradation of PC by phospholipase D, or by phospholipases A and C followed by phosphodiesterases may also contribute to intracellular choline, which can be reused for PC biosynthesis (Esko and Matsuoka, 1983). The contribution of PC-derived choline in cells incubated under normal conditions is questionable, however. It is possible that this may play an important role only under conditions of choline-depletion.

Choline is phosphorylated to phosphocholine by choline kinase (CK) by the transfer of the ATP γ-phosphate to choline (Wittenberg and Kornberg, 1953). This is generally believed to be the commited step in the pathway since phosphocholine and CDP-choline are obligate precursors of PC. CK, with a few exceptions, is a cytosolic enzyme. There are many reports claiming its purification to homogeneity from varied sources (Ishidate, 1989). Ishidate *et al.* (1984) have reported the purification of CK which has a molecular weight of 42,000 when subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE). CK is generally not considered to be a regulatory enzyme although it is, like CT, far from equilibrium i.e., the reaction catalyzed by CK is essentially irreversible in rat liver (Infante, 1977) and chick muscle cells (Sleight and Kent, 1980). This is because the enzymatic activity of CK does not usually correlate with the rate of PC biosynthesis. However, there are instances where the enzyme can be rate limiting (Reviewed by Pelech and Vance, 1984a;

Ishidate, 1989; Kent, 1990). There are recent questionable claims in the literature that CK regulates PC biosynthesis. Teergarden *et al.* (1990) claim that PC biosynthesis in *ras*-transfected C3H10T 1/2 rat fibroblasts was increased as evidenced by increased phosphocholine turnover rates. In addition, *in vitro* CK activity was increased approximately two-fold whereas particulate CT activity decreased by 50% compared to untransfected controls. In keeping with increased choline kinase activity the phosphocholine pool size in the *ras*-transfected cells had increased 3-4 fold over that in controls. Although continuous labeling experiments with [^{3}H]choline showed decreased label in lipids in addition to reduced PC mass, it was claimed by the authors that PC biosynthesis had *increased* possibly due to increased CK activity. More recently, Kaplan and Cohen (1991) have shown by ^{31}P NMR techniques that because phosphocholine levels in human lymphocytes were very low compared with other cell lines and increased when CT was inhibited by dapsone that CK was regulating PC synthesis in these cells. These reports indicate that CK is important in PC biosynthesis but fail to provide any conclusive evidence for assigning a regulator role to CK.

b) CTP:phosphocholine cytidylyltransferase - The second step in the CDP-choline pathway, the conversion of phosphocholine to CDP-choline, is catalyzed by CTP:phosphocholine cytidylyltransferase (EC 2.7.7.15) and is generally considered to regulate the overall rate of PC biosynthesis. Although Infante (1977) had suggested CK as being rate-limiting for PC biosynthesis, Vance and Choy (1979) summarized evidence from various studies that argued strongly for CT governing the rate of PC biosynthesis in most cell types. As such CT has an extremely important regulatory role in PC biosynthesis and is the target of intense investigation in various laboratories around the world and the subject of the reviews cited at the beginning of this chapter.

The CT catalyzed reaction was described in rat liver by Kennedy and Weiss (1956). It was subsequently shown in the same laboratory that the enzyme resided both in the cytosol and in microsomes (Wilgram and Kennedy, 1963). Vance and Pelech (1984) suggested that the cytosolic form of the enzyme was an inactive reservoir whereas the microsomal form was active. The authors reviewed data from various studies consistent with the hypothesis that CT translocates from the cytosol to the ER where it is activated by certain phospholipids.

CT may not always be activated by a translocation mechanism. Choy *et al.* (1980) showed that in poliovirus-infected Hela cells, the reaction catalyzed by CT was accelerated due to an increase in cytosolic CTP, in which case the availability of substrate regulated the pathway of PC biosynthesis.

There are instances in which the difference between CT translocation and increase in enzyme activity becomes somewhat indistinct. For example, it was shown in lymphocytes that low amounts of fatty acids increased PC biosynthesis with an increase in microsomal CT activity, but the decrease in cytosolic activity accounted for only 20% of the total activity in the membranes (Whitlon *et al.*, 1985). Fatty acid treatment of Type II cells isolated from rabbit fetal lung (Aeberhardt *et al.*, 1986) or adult rat lung (Burkhardt *et al.*, 1988) caused increased microsomal CT activity without a change in cytosolic activity. In contrast, Chander and Fisher (1988) showed increases in both microsomal and cytosolic CT activity in cells from adult lung upon treatment with fatty acids. It is difficult to draw any conclusions about CT translocation from the studies by Aeberhardt *et al.* and Burkhardt *et al.* because cytosolic CT activity in these studies was not assayed under optimal conditions i.e., lipids required for maximal CT activity were ommitted. In another study Mock *et al.* (1986) showed that stearic acid caused selective

activation of microsomal CT in hamster hearts possibly due to membrane modification by the fatty acid. It was unclear whether cytosolic CT had correspondingly decreased as the assays were not performed under optimal conditions (indeed the authors cautioned that translocation of CT could not be eliminated in these studies). Subsequent work by Hatch and Choy (1990) showed that accumulation of fatty acids in hypoxiated hamster hearts may have caused a translocation of CT from cytosol to microsomes. Weinhold *et al.* (1991) have shown that fatty acids stimulated PC biosynthesis in Hep G2 cells via activation of CT. Both cytosolic and microsomal fractions showed increased CT activity which was confirmed by immunochemical techniques.

In some cell types CT may also be regulated by its aggregation state. Weinhold *et al.* (1989) have demonstrated the presence of two forms of CT in the cytosol of lung, Hep G2 cells, A549 cells and alveolar type II cells, the so-called L-and H-forms. The L-form, which is a dimer of the MW 42,000 subunit, requires lipids for activity, whereas the aggregated H-form is a lipoprotein and relatively independent of lipids for activity. More recently, an investigation of the relationship between the cytosolic and membrane forms of CT in rat lung has revealed that oleic acid caused the translocation of the cytosolic L-form of CT to membranes where it was present as the H-form (Feldman *et al.*, 1990). In addition, oleic acid, which stimulated PC biosynthesis via activation of CT in Hep G2 cells, caused an increase in both cytosolic and membrane CT activities as evidenced by immunological techniques (Weinhold *et al.*, 1991). This increase was independent of protein synthesis. The increase in cytosolic CT was due to an increase in the H-form and it was concluded that fatty acids promoted the formation of native CT (H-form) from a pre-existing inactive form (undetected by immunochemical methods) in Hep G2 cells.

The subcellular distribution of CT has been proposed to be regulated by various mechanisms (reviewed most recently by Vance 1989 and 1990). Briefly, these include a reversible phosphorylation mechanism where dephosphorylation of CT causes it to translocate to membranes and phosphorylation reverts it back to the cytosol (Sanghera and Vance, 1989).

Fatty acids have been proposed to regulate CT binding to cellular membranes. In cultured hepatocytes, albumin-bound fatty acids caused a 2- to 3-fold stimulation of PC biosynthesis accompanied by translocation of cytosolic CT to microsomes (Pelech *et al.*, 1983). An even more dramatic effect was seen when HeLa cells were treated with fatty acids. Oleate (1 mM) stimulated a 5 to 10-fold stimulation of PC biosynthesis accompanied by a dramatic redistribution of cytosolic CT to membranes (Pelech *et al.*, 1984; Cornell and Vance, 1987a). The addition of albumin to the incubation media extracted the cellular fatty acids and reversed these effects.

Diacylglycerols and PC levels have also been proposed to modulate the subcellular distribution of CT. Choy *et al.* (1979) reported that increased amounts of diacylglycerol (DG) in Phospholipase C-treated or hypercholesterolemic rat liver cytosol promoted aggregation and activation of CT. In addition, CT was seen to bind DG-enriched phospholipase C-treated membranes and to egg PC vesicles containing 20-30 mol% DG (Cornell and Vance, 1987b). More recently, Kolesnick and Hermer (1990) have shown that DiC_8, a DG analogue, promotes purified CT to bind to boiled microsomes.

In another approach, intact cells were treated with exogenous phospholipase C which degraded PC on the plasma membrane to produce DG and phosphocholine (Sleight and Kent, 1980; 1983a; 1983b; 1983c; Wright *et al.*, 1985; Tercé *et al.*, 1988). In these studies, a reversible stimulation of PC biosynthesis and CT translocation to membranes was observed. However, it was

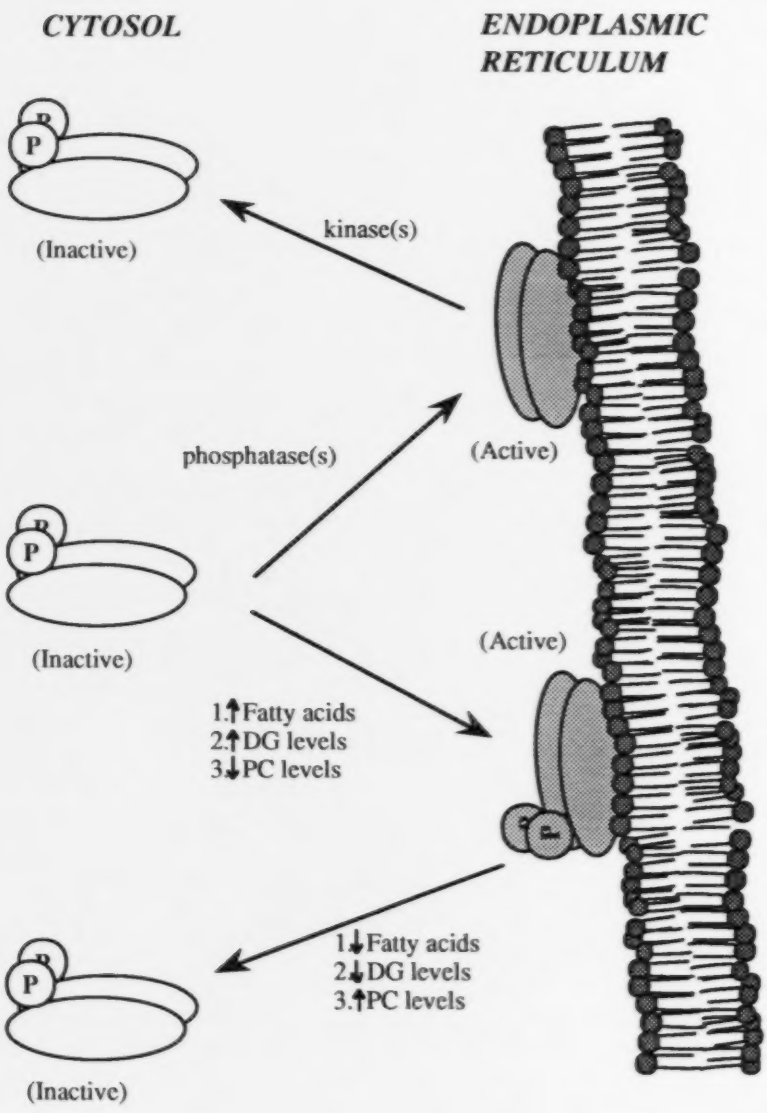
not clear whether the effects seen were due to increased DG or reduced PC levels. Evidence for a negative feedback role for PC came from studies on choline-deficient rat liver (Yao *et al.*, 1990) and rat hepatocytes (Jamil *et al.*, 1990) which indicated that PC levels could modulate the binding of CT to membranes; *decreased* PC levels caused CT to translocate to membranes, whereas *increased* PC levels caused CT to revert to the cytosol.

It is clear from these studies that, although CT in most cases regulates PC biosynthesis in a variety of cell types and conditions, the regulation mechanisms of the enzyme itself are very complex and dependent on the tissue or cell type under consideration and the conditions of incubation.

Fig. 3 summarizes the possible mechanisms of regulation of CT.

The full length cDNA of rat liver CT has been cloned (Kalmar *et al.*, 1990). It contains an open reading frame encompassing 367 amino acids. The predicted MW of 41,720 compares well with a MW of 42,000 estimated by SDS-PAGE of purified rat liver CT (Sanghera and Vance, 1989; Cornell, 1989). CT has been shown to exist as a dimer of the 42 KDa subunit (Cornell, 1989). The predicted amino acid sequence reveals several potential phosphorylation sites. Indeed, CT has been shown to be highly phosphorylated in HeLa cells (Watkins and Kent, 1990; Utal *et al.*, 1991). In addition, there do not appear to be any known consensus sequences for sites for covalent attachment of lipids. Rat liver CT has very little homology with protein kinase C (PKC), an enzyme which shares many regulatory properties with CT, for example, activation by anionic phospholipids, diacylglycerols and fatty acids, and inhibition by

Fig. 3. Proposed Mechanisms for Translocation of CT Between Cytosol and Endoplasmic Reticulum. It is postulated that CT exists in cytosol in an inactive, and phosphorylated form. The enzyme can be translocated to the endoplasmic reticulum (ER) by dephosphorylation, or the phosphorylated form could be bound to the membrane in the presence of increased fatty acids or DG, or decreased PC. Upon binding to membranes CT is activated by the lipids in the membrane. The association of CT with ER can be reversed by phosphorylation by a kinase such as cAMP-dependent protein kinase or reduction in the levels of fatty acid or DG, or increase in PC. (Modified from Vance, 1989).


CYTOSOL
ENDOPLASMIC RETICULUM
P
(Inactive)
kinase(s)
(Active)
phosphatase(s)
P
(Inactive)
(Active)
1.↑Fatty acids
2.↑DG levels
3.↓PC levels
1.↓Fatty acids
2.↓DG levels
3.↑PC levels
P
(Inactive)

sphingosine (Rando, 1988; Sohal and Cornell, 1990). An important feature of the predicted amino acid sequence of CT is a 58 residue amphipathic α-helix which is believed to mediate the association of CT with membrane lipids.

c) Cholinephosphotransferase - the final step in PC biosynthesis is the condensation of CDP-choline with DG, a reaction catalyzed by cholinephosphotransferase (CPT). CPT is an integral membrane protein localized largely on the ER (Van Golde *et al.*, 1971). To date complete purification of CPT has not been reported. The enzyme appears to be generally non-selective for its DG substrate. Ide and Weinhold (1982) showed that the PC formed was of the same molecular species as that of the substrate DG which was endogenously generated by incubating rat lung microsomes with various fatty acid precursors of DG.

1.1.2. *Methylation of Phosphatidylethanolamine*

The second route for PC synthesis involves *N*-methylation of PE. Successive transfer of three methyl groups from *S*-adenosyl-L-methionine (AdoMet) to PE is catalyzed in rat liver by a single enzyme, PE *N*-methyltransferase (PEMT), generating PC, the final product (Ridgway, 1989). Bremer and Greenberg (1960) discovered that the methyl groups of choline could be derived from *S*-adenosylmethionine by transmethylation. Conversion of PE to phosphatidylmonomethylethanolamine (PMME), the first methylation step is rate-limiting for this route (Bremer and Greenberg, 1961). In yeast two methyltransferases are involved in the pathway, one enzyme catalyzing the conversion of PE to PMME (phosphatidyl-*N*-monomethylethanolamine) and the other catalyzing all three reactions (Scarborough and Nyc, 1967). The purification of rat liver PEMT to homogeneity was reported by Ridgway and

Vance (1987) who showed that the enzyme was an 18.3 KDa protein as analyzed by SDS-PAGE.

PE methylation accounts for 20-40% of PC synthesis in the liver, the only organ where it might be quantitatively important (Sundler and Akesson, 1975). PE methylation activity was the highest in the liver and very low in other organs (Bremer and Greenberg, 1961; Vance *et al.*, 1982).

The literature on the subcellular distribution appears to be inconsistent. PEMT activities have been reported in the ER, and *cis*-Golgi whereas mitochondrial and plasma membrane associated PE methylation activities have been attributed to contamination from ER (reviewed by Ridgway, 1989).

1.1.3. *Are the CDP-Choline and PE Methylation Pathways Coordinately Regulated?*

The two major pathways for PC biosynthesis appear to be coordinately regulated. Unsaturated fatty acids which stimulate PC biosynthesis from choline in cultured rat hepatocytes (Pelech *et al.*, 1983) were found to inhibit PE methylation (Audubert *et al.*, 1984). Conversely, when PE methylation was inhibited by 3-deazaadenosine (DZA) (Chiang and Cantoni, 1979), a 2 to 3-fold increase in PC synthesis from choline was observed (Pritchard *et al.*, 1982). Similar results were observed in the livers of rats or hamsters treated with 3-DZA. In these tissues, although PE methylation was inhibited, overall phospholipid and PC content remained unchanged, presumably compensated for by the greatly increased incorporation of choline into PC (Chiang *et al.*, 1980). In addition, in the livers of choline-deficient rats where PC synthesis via the CDP-choline route is inhibited, there was a 2-fold elevation in *in vitro* PEMT activity (Schneider and Vance, 1979; Hoffman *et al.*, 1980) and was the direct result of increased PE levels in the membranes (Ridgway *et al.*, 1989).

More recently, PC biosynthesis in Madin-Darby Canine Kidney (MDCK) cells has been shown to be inhibited by a phospholipid analogue, hexadecylphosphocholine (Haase *et al.*, 1991). Interestingly, whereas the PC content in hexadecylphosphocholine-treated MDCK cells was decreased, PE mass was shown to be increased, indicating that PE methylation might compensate for some PC.

1.1.4. *Base Exchange*

Base exchange reactions have been defined by Kanfer as those which 'catalyze the incorporation of the aminoalcohols normally found in phospholipids into their corresponding phospholipids'. Kanfer also suggested that base exchange reactions are not responsible for net phospholipid synthesis but for remodeling of preexisting membrane phospholipids.

Phospholipase D and base-exchange activities are often considered together since they react with the same moiety of the phospholipid molecule. Base exchange enzymes catalyze the substitution of a free ethanolamine, serine, choline or inositol for a similar substituent on the preexisting phospholipids. Phospholipase D, on the other hand, is a hydrolytic enzyme which liberates the amino alcohol from intact phospholipids giving rise to phosphatidic acid. As reviewed by Kanfer (1989), base exchange activities have been studied in microsomes from various sources. It also appears to be a distinct enzyme activity from phospholipase D.

Suzuki and Kanfer (1985) reported the purification of serine and ethanolamine base exchange enzyme activity from solubilized rat brain microsomes to apparent homogeneity. The enzyme has a MW of 100,000 based on SDS-PAGE. Of the phospholipids tested only PE was capable of being the acceptor.

In contrast to PC biosynthesis the exchange of L-serine with the ethanolamine moiety of PE is believed to represent the principal route of PS biosynthesis in animals (Yavin and Zeigler, 1977). Mutant Chinese Hamster Ovary (CHO) cells auxotrophic for PS showed reduced serine base exchange activity (Kuge *et al.*, 1985, 1986a, 1986b). Studies indicated that these auxotrophs were deficient in cellular PS but not PC, suggesting that PC synthesis by exchange is of minimal importance. Choline exchange in CHO cells appears to be a futile process since PC is required as a lipid acceptor.

1.1.5. *PC Biosynthesis from Lysophosphatidylcholine*

The biosynthesis of PC from lysophosphatidylcholine (LPC) has been reviewed by Choy and Arthur (1989). Synthesis of PC from LPC serves not only to reduce the levels of LPC which is cytotoxic at high concentrations, but may also play a role in regulating the levels of free fatty acids (Irvine *et al.*, 1982) and for remodeling PC.

In studies with isolated and perfused Hamster hearts, Savard and Choy (1982) estimated that 14% of newly formed cardiac PC might be accounted for by reacylation of exogenous LPC. The *in vivo* formation of PC by the acylation of LPC has also been shown in the arterial wall (Portman and Illingworth, 1974).

In mammalian tissue LPC acylation is regulated by two ways:

1) an acyltransferase catalyzes the transfer of an acyl group from acyl CoA to LPC,

2) a transacylation reaction involves the transfer of an acyl group from another phospholipid directly to LPC without the release of free fatty acid.

The acyltransferase activities involved in the acylation of LPC to PC have been reported in the cytosolic, microsomal, mitochondrial and nuclear

fractions of a variety of tissues. Although acyltransferase activities have been reported in rat and pig livers, lung and brain, it is unclear to what extent they may contribute to overall PC biosynthesis. As mentioned above, 14% of the PC may be derived from LPC acylation in hamster heart. This pathway may be significant in this organ as a method of regulating the levels of free fatty acids which are the principal sources of energy in the cardiac tissue.

1.1.6. *Precursors of Glycerolipid Synthesis*

The regulation of glycerolipid synthesis has been reviewed in detail by Brindley (1985). Phosphatidic acid (PA) plays a central role in glycerolipid synthesis. It can be channeled to the synthesis of acidic phospholipids, phosphatidylinositol, phosphatidylglycerol and diphosphatidylglycerol via CDP-diacylglycerol, or PA can be dephosphorylated by phosphatidate phosphohydrolase (PAP) to DG. DG is a precursor for zwitterionic phospholipids, PC, PE and for triacylglycerol (TG). In most tissues PA is thought to be derived from the metabolism of glycerol-3-phosphate formed by glycolysis. In the liver glycerol-3-phosphate is also obtained by phosphorylation of glycerol and can be acylated to 1-monoacylglycerol-3-phosphate (or lysophosphatidic acid) by glycerolphosphate acyltransferase. PA can also be synthesized by acylation of dihydroxyacetone phosphate (DHAP) by glycerolphosphate acyltransferase. In rat liver slices, it was shown that 50-70% of glycerolipid synthesis occured by acylation of DHAP (Manning and Brindley, 1972). Similar estimations were obtained in lung type II cells (Mason, 1978) and BHK-21 and BHK-TS-a/lb-2 cells (Pollock *et al.*, 1976).

1.1.7. *Phosphatidate Phosphohydrolase*

Phosphatidate Phosphohydrolase (PAP) plays an important role in glycerolipid synthesis as it can dephosphorylate PA to provide DG, a precursor for the synthesis of zwitterionic phospholipids, PC and PE and for TG. The purification of the enzyme has not been reported to date. Much of the initial characterization of PAP activities has been reported from the laboratory of Dr. David N. Brindley. PAP has been reported in plasma membrane, lysosomes, mitochondria, ER and cytosol (Brindley, 1984 and 1988). There are at least two different PAP activities in the liver (Martin *et al.*, 1991; Jamal *et al.*, 1991). The cytosolic PAP activity is dependent on Mg^{2+}, is inhibited by thiol group reagents such as *N*-ethylmaleimide and can translocate to the ER (Brindley, 1984 and 1988) and mitochondria (Freeman, 1989) in response to fatty acids. This type of regulation of PAP is consistent with a regulatory role in glycerolipid synthesis.

The second PAP activity has distinct properties from the one just described. The second PAP has a plasma membrane location, is not inhibited by *N*-ethylmaleimide, and is not stimulated by Mg^{2+} (Jamal *et al.*, 1991). The plasma membrane location of the enzyme is consistent with a role in signal transduction. The plasma membrane PAP may play a major role in the dephosphorylation of PA following agonist- or phorbol ester-stimulated phospholipase D hydrolysis of phospholipids. This has a two-pronged implication; not only would the plasma membrane PAP contribute to the second and sustained phase of DG in agonist- or phorbol ester-stimulated cells, but would do so by preventing the accumulation of PA thereby making the PA signal short-lived. Both DG and PA are considered second messengers but their specific targets, besides the well known activation of protein kinase C by DG,

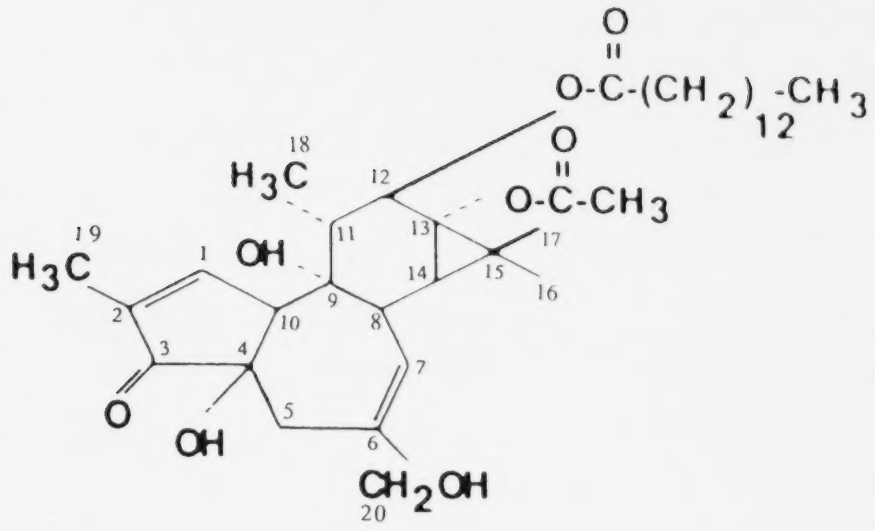
are largely unknown. In addition, CT can be targeted by TPA-stimulated DG (Utal *et al.*, 1991).

1.2. PHORBOL ESTERS; AN HISTORICAL PERSPECTIVE

Phorbol esters are a class of potent tumor-promoters which exert profound effects on cell growth and proliferation. In 1941, Berenblum while investigating the relatedness of certain skin irritants to their role in carcinogenesis at Oxford University in England, described the oil from croton seeds as 'cocarcinogenic'. Croton seeds are obtained from *Euphorbiacea croton tiglium L.*, a leafy shrub native to southeast Asia. The studies of Berenblum stemmed from investigations begun in 1936 to determine whether 'an optimal degree of irritation was necessary for carcinogenesis'. Substances such as xylene, turpentine and croton oil were tested in the now classic 'Berenblum Experiments' to ascertain whether certain substances, without themselves being carcinogenic, could 'augment the tumor-producing action of a carcinogenic agent when the degree of irritation by the latter was insufficient'. A petroleum ether extract of croton oil (called croton residue) was found to possess even greater cocarcinogenic action than total oil. It was more than twenty years before the successful isolation and elucidation of the chemical structure of the tumor-enhancing constituents of croton oil were reported (Van Duuren *et al.*, 1963; Van Durren and Orris, 1965; Hecker, 1968). The croton residue was reported to have eleven compounds with similar molecular structure and these were described as diesters of phorbol, the parent compound (Hecker, 1968). In the same report, an investigation of some synthetic 12-*O*-acyl-phorbol-13-acetates as a function of fatty acid chain length, showed that a chain length of 14 carbons at position 12 conferred the greatest cocarcinogenic activity on the phorbol ester.

Subsequently, other biological effects of phorbol and four synthetic diesters of phorbol were correlated with their tumor-promoting potentials (Baird and Boutwell, 1971; Baird, Sedgwick and Boutwell, 1971). 12-*O*-tetradecanoylphorbol 13-acetate (TPA) and phorbol-12,13-didecanoate were found to be the most effective in tumor-promotion as well as stimulation of DNA, RNA and protein synthesis in mouse epidermis. Phorbol, the parent compound, was ineffective. Fig. 4 shows the structure of TPA, biologically the most potent phorbol ester.

Studies on the effects of TPA on phospholipids were initiated after it was rationalized that TPA most probably interacted with membrane sites which controlled DNA synthesis and cell division (Süss *et al.*, 1971; Rohrschneider *et al.*, 1972), particularly as studies by Sivak *et al.* indicated that cell membranes are important targets for phorbol esters (Sivak *et al.*, 1969; Sivak *et al.*, 1972; Sivak, 1972). Rohrschneider *et al.* (1972) and others showed that TPA stimulated the incorporation of ^{32}Pi (Balmain and Hecker, 1974) and [^{3}H]choline (Rohrschneider *et al.*, 1973) into phospholipids, the greatest effect being on radiolabeling of PC. This effect was independent of protein or RNA synthesis and was deduced to be due to pre-existing enzymes. These studies also correlated positively the stimulation of phospholipid synthesis with the tumor-promoting ability of different phorbol esters. Subsequent studies with bovine lymphocytes (Wertz and Mueller, 1978) and HeLa cells (Kinzel *et al.*, 1979) confirmed the potent stimulatory effect of phorbol esters on PC metabolism. The significant correlation between the stimulation of [^{3}H]choline incorporation in cell cultures and the tumor-promoting abilities of phorbol esters led Kinzel *et al.* (1979) to suggest the use of [^{3}H]choline incorporation into cell lines as a measure of the potency of tumor promoting substances.



12-*O*-TETRADECANOYLPHORBOL 13-ACETATE

Fig. 4. Structure of the Tumor Promoter, 12-*O*-Tetradecanoylphorbol 13-Acetate (TPA), (modified from Welsh and Cabot, 1987; originally put foward by Hecker, 1968)

1.3. ACTIVATION OF PROTEIN KINASE C BY PHORBOL ESTERS

1.3.1. *Protein Kinase C as a Phorbol Ester Receptor*

Protein kinase C (PKC) was discovered in the laboratory of Dr. Yasutomi Nishizuka at Kobe University in Japan as a novel proteolytically-activated, cyclic nucleotide-independent protein kinase (Inuoe *et al.*, 1977; Takai *et al.*, 1977) and was subsequently shown to be dependent on Ca^{2+} and acidic phospholipids *in vitro* (Takai *et al.*, 1979b). The simultaneous discovery that this kinase was activated by diacylglycerol (DG), a product of phosphatidylinositol-4,5-bisphosphate (PIP_2) turnover (Takai *et al.*, 1979a; Kishimoto *et al.*, 1980) suggested that protein kinase C had an important role in this signal transduction pathway. Available evidence suggested that phorbol esters exerted their effects at the cell surface by binding to specific receptors (Driedger and Blumberg, 1980; Shoyab and Todaro, 1980; Jaken *et al.*, 1981). However, it was not until PKC was identified as the major cellular receptor for phorbol esters, that a role for PKC in tumorigenesis was established (Ashendel *et al.*, 1983a, 1983b, 1983c; Leach *et al.*, 1983; Neidel *et al.*, 1983).

One of the most important observations was that TPA could activate PKC by substituting directly for DG without involving PIP_2 breakdown (Castagna *et al.*, 1982). Subsequently, Sharkey *et al.* (1984) showed that DG interacts with PKC at the same site as phorbol esters. Activation of PKC by diacylglycerols and other lipids has been reviewed (Bell, 1986; Nishizuka, 1986; Bell and Burns, 1991).

1.3.2. *Translocation of PKC*

PKC was shown to be regulated by movement between the cytosol and plasma membrane by Kraft and Anderson, who initially showed that phorbol ester treatment of EL4 thymoma cells caused a 90% decrease of cytosolic

phorbol ester receptor (Kraft and Anderson, 1982), and subsequently showed that TPA caused a concomitant increase of PKC in the plasma membranes of parietal yolk sac (PYS) cells (Kraft and Anderson, 1983). At the same time Ashendell *et al.* (1983) also showed that PKC could move between the cytosol and cell membranes. Since then the phorbol ester-induced rapid and quantitative translocation of PKC from cytosol to membranes has been documented in almost every cell type under investigation.

1.3.3. *Synergistic Activation of PKC by Ca^{2+} and Phorbol Esters*

The synergistic effect of Ca^{2+} and phorbol esters on PKC translocation and activation was investigated and a model proposed in which Ca^{2+}, which was ineffective by itself, recruited PKC to the plasma membrane, thus priming the system for activation by phorbol esters (Wolf *et al.*, 1985; May *et al.*, 1985). Ganong *et al.* (1986) hypothesized that DG interacts with Ca^{2+} to stabilize the membrane-bound PKC. Similarly, Siess and Capetina (1988) have suggested that Ca^{2+} may prime the binding of PKC to human platelet plasma membranes, placing it in a strategic position for activation by phorbol esters. Indeed, concentrations of phorbol esters that by themselves caused only slight PKC activation, elicited rapid and large PKC stimulations when added in combination with low concentrations of calcium-mobilizing agents.

Snoek *et al.* (1988) have proposed a model in which the initial binding of PKC to membranes is electrostatic involving calcium bridges. DG or phorbol esters induce tighter binding to membranes favouring hydrophobic interaction and facilitating activation of PKC. This model supports the findings that PKC may be able to interact with membranes in either a tightly- or loosely-bound fashion (Gopalakrishna *et al.*, 1986; Bazzi and Nelsustuen, 1989).

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1.3.4. *Down-Regulation of PKC*

Prolonged treatment of cells with phorbol esters causes down-regulation of PKC. A down-modulation of [^{3}H]phorbol-12,13-dibutyrate binding sites upon prolonged treatment of GH_4 C1 rat pituitary cells with phorbol esters was first shown by Jaken *et al.* (1981). It was subsequently shown that membrane-associated PKC was susceptible to limited proteolysis by calpain (Kishimoto *et al.*, 1983), and that prolonged treatment of many cell types with phorbol esters caused a total disappearence of cellular PKC activity (Rodriguez-Pena and Rozengurt, 1984). This disappearence of PKC was confirmed by Ballester and Rosen (1985) who showed a complete loss of immunodetectable PKC from GH_3 cells after prolonged treatment with phorbol esters.

Phorbol esters were shown to have no effect on either the synthetic rate of PKC (Woodgett and Hunter, 1987) or the levels of PKC-specific mRNA transcripts (Young *et al.*, 1987; Mizuguchi *et al.*, 1988). It has been suggested that calpain may be the protease responsible for PKC degradation, since leupeptin, a calpain inhibitor, prevented the loss of PKC by TPA (Ito *et al.*, 1988).

1.3.5. *PKC Heterogeneity*

Structural information on PKC first became available when the protein was cloned. Oligonucleotide probes based on a partial amino acid sequence of purified bovine brain PKC were used to obtain cDNA clones from bovine brain cDNA libraries (Parker *et al.*, 1986). The complete primary structure of PKC was elucidated and revealed cysteine-rich domains followed by a putative Ca^{2+}-binding domain at the amino terminus. Substantial homology with other protein kinases was revealed at the carboxyl terminus. Based on this study, it

was shown that there was considerable heterogeneity in the so-called PKC family, and Northern and Southern hybridization analyses suggested an even greater genetic complexity than was revealed in these studies. To date seven isozymes of PKC, namely α, β (βI plus βII), γ, δ, ε and ζ, have been discovered. This heterogeneity and its implication in cellular regulation has been reviewed by Nishizuka (1988) and Kikkawa *et al.* (1989). These isozymes have dissimilar cofactor requirements, bind to phorbol esters with different affinities (Burns *et al.*, 1990) and are differentially translocated to membranes (Fournier *et al.*, 1989). The isozymes also have different susceptibilies to proteolytic degradation *in vitro* and in intact cells treated with TPA (Huang *et al.*, 1989). Moreover, different cell lines show different patterns of TPA-induced downregulation of PKC (Adams and Gullick, 1989; Robes-Flores *et al.*, 1991). It is also known that some PKC sub-types are more resistant to down-regulation in TPA-treated cells than others (Strulovici *et al.*, 1991). Clearly PKC sub-types have distinct properties from each other and the elucidation of their roles in different cell types is a logical progression in their study.

Indeed, a baculovirus-insect cell expression system developed in the laboratory of Dr. Robert Bell (Duke University, North Carolina) for the expression of different PKC sub-types appears to be a powerful tool for the characterization of the individual proteins (Burns *et al.*, 1990). This approach in studying individual sub-types of PKC is extremely useful given the difficulties in the consistent separation of the proteins from each other.

One of the most important challenges in the study of PKC is finding the natural cellular substrates for the various sub-types.

More recently, an interesting twist was added to the study of PKC regulation by Pelech *et al.* (1991). The authors showed that in rabbit platelets treated with TPA there was conversion of PKC-β from being calcium- and lipid-

dependent for histone I phosphorylation to forms that had different elution characteristics upon Mono Q column chromatography and correspondingly different co-factor requirements. Treatment of two of these forms of PKC with alkaline phosphatase resulted in a protein with the characteristics of the original form, suggesting that the phosphorylation state of PKC has important implications in the regulation of its activity.

1.4. TPA-STIMULATED PC BIOSYNTHESIS VIA THE CDP-CHOLINE PATHWAY

1.4.1. *TPA Stimulates PC Biosynthesis in a Variety of Cell Types*

The effect of phorbol esters (or croton oil, before purification and characterization of its cocarcinogenic constituents in the 1960s) on phospholipid metabolism has been under intense investigation ever since it was hypothesized that phorbol esters mediated their pleiotropic effects via the outer cell membrane (see section 1.2). Studies in the early 1970s showed that the increased incorporation of [^{3}H]choline into PC was one of the largest known responses to TPA and the stage was set for the study of PC biosynthesis stimulated by TPA via the CDP-choline route, particularly with respect to the role of this event in carcinogenesis (Rohrschneider *et al.*, 1972; Rohrschneider *et al.*, 1973; Balmain and Hecker, 1974). Subsequently, other studies confirmed the increased incorporation of [^{3}H]choline or ^{32}Pi due to TPA into the phospholipids of bovine lymphocytes (Wertz and Mueller, 1978), Hela cells (Kinzel, 1979; Paddon and Vance, 1980; Guy and Murray, 1982) human myeloid leukemia cells (Cabot *et al.*, 1980), cultured myoblasts (Grove and Schimmel, 1980), the neuroblastoma-glioma hybrid cell line, NG108-15

(Liscovitch, 1986), GH_3 pituitary cells (Kolesnick and Paley, 1987; Kolesnick, 1989), and more recently by Kiss *et al.* (1991) in rat-1 fibroblasts, NIH 3T3 fibroblasts and A-*raf*-transformed 3T3 fibroblasts. Wertz and Mueller (1978) and Kinzel *et al.* (1979) also showed that this response, as it was unaffected by inhibitors of RNA and protein synthesis, was due to pre-existing enzymes.

Although a wide variety of agents have been shown to stimulate PC biosynthesis mainly by activation of CTP:phosphocholine cytidylyltransferase (CT), the rate-limiting enzyme in the CDP-choline pathway (see section 1.1.1 b), the mechanisms immediately preceeding CT activation are unclear. Many hormonal and growth factor effects appear to be due to PKC activation caused by DG production via the phosphatidylinositol signal transduction pathway (Berridge *et al.*, 1987). Since phorbol esters are now commonly used to probe PKC function because of their structural similarity to DGs, the literature review in this section will be confined exclusively to the effects of phorbol esters on PC biosynthesis and references will be made to exogenous DG-mediated events where appropriate.

1.4.2. *The Mechanism of Stimulation*

The elucidation of the mechanism whereby phorbol esters stimulate PC biosynthesis was begun in the laboratory of Dr. Dennis Vance. It was shown that TPA stimulated PC biosynthesis in Hela cells by increasing the rate of the reaction catalyzed by CT without affecting the *in vitro* activity or kinetic parameters of the enzyme (Paddon and Vance, 1980). Subsequently Hill *et al.* (1984) confirmed these observations in TPA-treated rat skeletal myoblasts. Further investigation revealed that there was a 2.3-fold activation of CT activity in the homogenates of TPA-treated Hela cells (Pelech, Paddon and Vance, 1984). Subcellular fractionation studies showed that TPA activated CT

by causing it to translocate from an inactive cytosolic form to an active membrane-associated form. The mechanism of TPA-elicited CT translocation was studied by Cook and Vance (1985) and shown not to be due to the release of free fatty acids. Previously, fatty acids had been shown to translocate cytosolic CT to membranes in rat hepatocytes (Pelech *et al.*, 1983) and HeLa cells (Pelech *et al.*, 1984). Cook and Vance (1985) also showed that CT could not be phosphorylated by PKC using partially purified preparations of the enzymes. Subsequently, Jamil, H. and Vance, D.E. (unpublished observations) were able to reproduce a similar result with pure PKC and CT. Watkins and Kent (1990) showed for the first time by immunoprecipitation methods that the phosphorylation state of cytosolic CT was unchanged by TPA-treatment of HeLa cells. This study was, however, unable to reproduce the earlier studies of Pelech *et al.* (1984) showing TPA-mediated CT translocation to membranes. The authors were also unable to visualize cytosolic CT by immunoblotting methods. Subsequently, Utal *et al.* (1991) resolved the issue by demonstrating that CT translocation to membranes did indeed occur upon TPA-treatment of HeLa cells, both by activity measurements and immunoblotting methods. The authors also confirmed the lack of a change in the phosphorylation state of cytosolic CT as reported earlier by Watkins and Kent (1990). It appears from these studies that fatty acid accumulation or a direct phosphorylation of CT as mechanisms of translocation to membranes in response to TPA can be ruled out.

TPA has been shown to stimulate PC biosynthesis in a variety of cell types and generally appears to be due to activation of the rate-limiting enzyme for PC biosynthesis, CT. While it is generally accepted that activation of CT by TPA is due to translocation of the enzyme to membranes, the mechanism of this translocation was unknown. Guy and Murray (1982) showed that PC hydrolysis

(as evidenced by radiolabeled choline release from cells prelabeled with [^{3}H]choline) preceeded stimulation of PC biosynthesis in HeLa cells treated with TPA, and that this effect could be mimicked by treating cells with exogenous phospholipase C. The authors suggested that TPA stimulated PC biosynthesis in a manner similar to that of phospholipase C. Earlier, Grove and Schimmel (1981) had shown that 30 min treatment of chick embryo differentiated myoblasts with TPA caused a two-fold increase in DG which was not due to *de novo* synthesis but was derived from pre-existing phospholipids located in the plasma membrane fraction. The authors suggested that alterations in lipid metabolism could be secondary to increased DG generation. It was subsequently shown by gas liquid chromatography (GLC) analysis that the fatty acid composition of the newly-derived DG was similar to that of PC which indicated that TPA might stimulate PC hydrolysis via a membrane-associated phospholipase C to generate DG which could then be metabolized back to phospholipids (Grove and Schimmel, 1982). It was unclear until recently how DG, a hydrolysis product of PC could stimulate PC biosynthesis when Utal *et al.* (1991) provided evidence showing that the production of DG due to TPA-treatment of HeLa cells caused the translocation of CT to membranes and thereby stimulated PC biosynthesis.

1.4.3. *TPA May Not Always Stimulate PC Biosynthesis*

There are reports in the literature that suggest that TPA-stimulation may not always increase PC biosynthesis (Plagemann and Estensen, 1980; Muir and Murray, 1988; Cook *et al.*, 1989). Plagemann and Estensen (1980) could show stimulation of [^{3}H]choline incorporation into HeLa cells within 60 min but not in 3T3 and other cultured cell lines with various concentrations of TPA for upto 6 or 16 h. Kinzel *et al.* (1979) showed that TPA *could* stimulate

incorporation of [^{3}H]choline into 3T3 cells, an effect that has since been reproduced (Muir and Murray, 1987; Takawa *et al.*, 1987; Kiss *et al.*, 1991). It is unclear why Plagmann and Estensen were unable to show a similar result.

In another study Muir and Murray (1988) concluded that treatment of human platelets with phospholipase C or the DG analogue, DiC_8 could stimulate the incorporation of ^{32}Pi into PC over 20 min of incubation, but not with TPA or the agonist, thrombin. It is possible that the authors failed to take into account the time factor; whereas phospholipase C and DiC_8 could exert their effects almost immediately, TPA and thrombin would require a certain lag period (presumably to activate the pathways for the generation of DG) before their effects would be apparent. Therefore, the 20 min of incubation in the study by Muir and Murray (1988) would be insufficient for TPA and thrombin to manifest themselves. Indeed, a time-course study of TPA-stimulated PC biosynthesis in HeLa cells showed a 30 min lag before increased incorporation of [^{3}H]choline into PC became apparent (Utal *et al.*, 1991).

Cook *et al.* (1989) showed that TPA was ineffective in stimulating PC biosynthesis in the neuroblastoma cell line (N1E-115). The significance of this is unclear as in the same study TPA was able to stimulate PC biosynthesis 1.5 to 3-fold in Glioma (G_6) cells and to an intermediate extent in the hybrid cell line, NG108-15. The difference would appear to be at at the level of PKC activation or PC hydrolysis (parameters not investigated in this study) since the PC biosynthetic machinery of the cells was operative as seen by oleic acid stimulation of PC biosynthesis in all three cell lines.

1.4.4. *Does TPA Cause PC Hydrolysis to Trigger its Own Synthesis?*

Intracellular signalling by PC hydrolysis products formed due to treatment of cells with phorbol esters or various agonists, and the mechanisms

mediating PC hydrolysis are the subjects of comprehensive reviews by Exton (1990) and Billah and Anthes (1990), respectively. The objective of this section, therefore, is not to list the reports that have already been compiled in these reviews but to try and understand instances which might diverge from the present consensus in the literature.

It is now generally accepted that PC biosynthesis is triggered by PC hydrolysis presumably by signalling from the hydrolysis products themselves. The mechanisms whereby these hydrolysis products may stimulate PC biosynthesis are only beginning to be understood. Treatment of various cells with phorbol esters invariably causes PC (and PE) hydrolysis where measured. Various methods have been used to show that TPA-treatment of cells causes PC hydrolysis. These methods have generally been to prelabel cellular PC with appropriately labeled precursors followed by analyses of intra- and extra-cellular metabolites formed upon incubation of the cells with TPA (Reviewed by Exton, 1990; Billah and Anthes, 1990).

It is apparent from many of these studies that PC hydrolysis and biosynthesis are intimately linked, and where studied it is apparent that the former preceeds the latter. A recent report has questioned this linkage between hydrolysis and stimulation of biosynthesis (Kiss *et al.*, 1991). This study has compared the extent of TPA-mediated release of radiolabeled hydrolysis products of PC and PE in various cell lines with the extent of TPA-stimulated [^{3}H]choline-incorporation. Since the differences observed in PC hydrolysis do not correlate quantitatively with stimulation of PC biosynthesis, the authors conclude that PC synthesis and hydrolysis are not linked. However, the authors appear to have overlooked several things:

(1) To analyze seriously and quantitatively correlate these two events, it is imperative that the specific radioactivity of the metabolites under

investigation be considered. This may be particularly significant if correlations are to be made among different cell lines.

(2) The authors were unable to show an increase in DG mass in TPA-treated 3T3 cells inconsistent with a previous report by Takawa *et al.* (1987) which showed substantial (at least two-fold) increase in the DG content of TPA-treated 3T3 cells

(3) In some instances the data has been reported in percentages rather than absolute values making comparisons among different cell lines impossible

(4) A comparison has been made between 3T3 and *A-raf*-transformed 3T3 cells with respect to TPA-stimulated PC hydrolysis and biosynthesis. *Ras*-transformed 3T3 cells have previously been shown to have elevated levels of DG to start with (Wolfman and Macara, 1987; Lacal *et al.*, 1987), higher steady state levels of phosphocholine (Lacal *et al.*, 1987) and fewer phorbol ester binding sites (Wolfman and Macara, 1987). These differences were not taken into account by the authors, making the interpretation of their results difficult.

Due to the points mentioned above the conclusions arrived at in the report by Kiss *et al.* (1991) are questionable until more detailed studies are undertaken to demonstrate uneqivocally the relationship between PC hydrolysis and biosynthesis.

In another report, Cabot *et al.* (1988) have shown that phorbol esters stimulate PC hydrolysis in REF 52 cells but not in the transformed deratives. Although not addressed by the authors this difference can be explained on the basis of the observations of Wolfman and Macara (1987) and Lacal *et al.* (1987) that elevated levels of DG mass in transformed cells may downregulate PKC to some extent and therefore, make them less sensitive or unresponsive to

phorbol ester treatment. Indeed the data of Cabot *et al.* (1988) shows elevated steady-state levels of [2-^{3}H]glycerol-labeled DG in the transformed cells. PC biosynthesis was not studied in this report, making it difficult to draw conclusions on the association between PC hydrolysis and synthesis. However it would be logical to speculate that the transformed derivatives would not respond to TPA-stimulation of PC biosynthesis as they would be expected to have a high rate of PC biosynthesis *per se* and low levels of PKC.

In conclusion, the data in the existing literature supports the view that the hydrolysis products of PC stimulate its biosynthesis. However once PC biosynthesis has been triggered it is possible that various catabolic pathways may be activated to restore homeostasis. Studies on this should provide further insight into the balancing cellular mechanisms that come into play. Indeed recent studies by Tijburg *et al.* (1991) and Tercé *et al.* (1991) provide evidence that PC catabolism may be regulated by increased PC biosynthesis, presumably by PC levels themselves.

1.5. STIMULATION OF PC BIOSYNTHESIS BY TPA VIA PE METHYLATION

The contribution of the PE methylation pathway to PC appears to be quantitatively important only in the liver (see section 1.1.2.). However recent evidence from Kiss (1990) suggests that in NIH 3T3 cells TPA-treatment results in greater hydrolysis of PC derived from methylated PE than of PC derived from the CDP-choline route. An earlier report by Liscovitch (1987) also showed a release of [^{3}H]choline from TPA-treated NG108-15 cells prelabeled with [^{3}H]methionine. Kiss (1990) showed that TPA-treatment of 3T3 cells prelabeled with [^{14}C]ethanolamine caused a greater than 70% release of [^{14}C]choline from the [^{14}C]ethanolamine-labeled PC, compared to a 4.2% release

of [^{14}C]choline from cells prelabeled with [^{14}C]choline. In the same study it was shown that TPA caused hydrolysis of 50% of newly derived PC from PE methylation in 3T3 cells prelabeled with exogenous [^{32}P]PE. These results have important implications. They suggest that

(1) TPA may preferentially cause the hydrolysis of a very specific pool of PC,

(2) although a quantitatively minor route for PC biosynthesis, PE-derived PC may constitute a metabolically active pool of PC, and

(3) PE methylation may be activated under conditions requiring PC synthesis in non-hepatic cell lines as in the case of TPA-stimulation via PC hydrolysis.

PE methylation in tissues other than the liver has not attracted attention because of its relatively minor contribution to PC biosynthesis. The findings of Kiss (1990) may, however, shift somewhat the focus of TPA-mediated effects on PC metabolism towards PE methylation.

The literature on the effects of TPA on PE methylation is scarce. Kelley (1987) reported that TPA-stimulated phospholipid methyltransferase activity in rat adipocytes by approximately 1.4-fold. In addition, Villaba *et al.* (1987) reported that a purified preparation of PEMT (MW 50,000) was phosphorylated by bovine brain PKC, and under these conditions PEMT activity was stimulated two-fold. In the same study tryptic-peptide mapping of the phosphorylated protein using high performance liquid chromatography revealed that the protein was phosphorylated at two different sites, predominantly at serine. The work of Ridgway and Vance (Ridgway, 1989), however, indicates that the 50 kDa protein is a contaminant and that the purified PEMT exhibits a MW of 18,300 on SDS-PAGE. In light of these findings reports claiming direct phosphorylation of PEMT need to be reevaluated. However, it *is* significant that incubation of PKC with the PEMT preparation of Villaba *et al.* (1987) stimulated PEMT activity and entirely consistent with the report by Kiss (1990)

which shows that TPA stimulated the hydrolysis of PE-derived PC, suggesting that PE methylation may also be stimulated. PE synthesis is also stimulated by TPA-treatment of myoblasts (Hill *et al.*, 1984), 3T3 fibroblasts (Takuwa *et al.*, 1987) and HL60 cells (Kiss *et al.*, 1989). These reports are consistent with a mechanism whereby increased PE would be available for methylation by PEMT to produce PC, whose hydrolysis is stimulated by TPA.

Although these reports appear to explain the results of Kiss (1990), it is difficult to explain them in the context of coordinate regulation of PC biosynthesis by the two main pathways (section 1.1.3), or the observations of Cassileth *et al.*, (1981) in HL60 cells where TPA caused increased incorporation of radiolabeled choline and coordinately decreased incorporation of radiolabeled methionine into cellular phospholipids. Moreover, in this study TPA stimulated PC biosynthesis by only 50%, making it difficult to interpret the results.

1.6. PKC-DEPENDENT AND -INDEPENDENT EFFECTS OF TPA AND OTHER DG ANALOGUES

It is evident thus far that TPA, one of the most potent phorbol esters known, is extensively employed as a probe for PKC function. TPA is believed to be effective because it is a structural analogue of DG, the natural lipid co-factor for PKC. TPA is also relatively metabolically stable in tissue culture as shown by Welsh and Cabot (1987) in HL60 cells where only 1% of [20-^{3}H]TPA was metabolized after one hour of incubation whereas other 1,2-diacyl analogues of DG were short-lived and rapidly metabolized (e.g., 95% of 1-oleoyl-2-acetyl-*sn*-glycerol was metabolized to phospholipids, triacylglycerol or monoacylglycerol within the one hour of incubation). This degree of

stability makes TPA particularly competent in sustaining biological signals normally governed by short-lived diacylglycerols.

There is overwhelming consensus in the literature that TPA exerts its effects exclusively via PKC activation. While this consensus is unchallenged, certain biological effects that are generally mediated by TPA via PKC activation (and mimicked by other DG analogues) are sometimes said to be PKC-independent. The reason for suggesting that certain biological events can be operative via PKC-independent pathways is that high levels of exogenous DG analogues can bring about similar effects in instances where PKC is inhibited or is depleted in cells by prolonged treatment with phorbol esters. Since TPA-activation of PKC generally results in accumulation of intracellular DG it may be incorrect in many cases to refer to DG-mediated events as 'PKC-independent', since this implies more complex mechanisms than are warranted. Supplying cells with the product of PKC activation does not constitute a different pathway, e.g., in rat adipocytes, TPA, exogenously added DGs and endogenously generated DGs by phospholipase C-treatment could mimic the stimulation of glucose uptake by insulin (Stälfors, 1988). Long chain DGs which are unable to activate PKC were also able to stimulate glucose uptake. It would appear that supplying a PKC activation product itself, namely DG, a TPA-effect was simulated.

The list of so-called PKC-independent effects of DG include the following: stimulation of PC biosynthesis (Kolesnick and Paley, 1987; Liscovitch *et al.*, 1987), stimulation of sphingomyelin hydrolysis (Kolesnick, 1987), activation of platelets (Ashby *et al.*, 1985), inhibition of granulosa cell maturation (Shinohara *et al.*, 1985), maturation of HL60 cells (Kreutter *et al.*, 1985), superoxide production in neutrophils (Ozaki *et al.*, 1986), glucose uptake in adipocytes (Stälfors, 1988), mobilization of intracellular calcium in platelets

(Brass and Laposata, 1987) and stimulation of melanogenesis in UV-irradiated human melanocytes (Friedman, 1990). In most of these studies the differences between TPA and other DG analogues can be explained quite simply.

1.6.1. *PKC-Independent Stimulation of PC Biosynthesis*

Reports claiming PKC-independent stimulation of PC biosynthesis can be easily explained on the basis of intracellular DG levels. TPA and the DG analogues DiC_8 and OaG, were shown to stimulate the incorporation of [^{3}H]choline into PC in GH_3 rat pituitary cells (Kolesnick and Paley, 1987) and in NG108-15 neuroblastoma x glioma hybrid cells (Liscovitch *et al.*, 1987). In these studies, inactivation of PKC either by its down-regulation or by the use of H-7, a PKC inhibitor, abolished the stimulation of PC biosynthesis by TPA but not that by the DG analogues. Moreover, treating the cells simultaneously with both TPA and DiC_8 resulted in an additive effect (Liscovitch *et al.*, 1987; Kolesnick, 1990), which suggested according to the authors, to be due to different pathways. Subsequently, it was shown that CT, the rate-limiting enzyme in PC biosynthesis is translocated to membranes (and thereby activated) in response to increased intracellular DG levels (Utal *et al.*, 1991). Kolesnick (1990) has also provided *in vitro* evidence for translocation of CT by DiC_8. Therefore, it would appear that both DiC_8- and TPA-mediated stimulation of PC biosynthesis are, in effect, via the same pathways, that of CT activation due to increased levels of DG.

1.6.2. *The Differences Between DG Analogues on Sphingomyelin Metabolism*

DiC_8-treatment of GH_3 rat pituitary cells prelabeled to equilibrium with [^{3}H]choline caused a 42% decrease in labeled sphingomyelin compared to

controls after one hour of treatment, suggesting hydrolysis of sphingomyelin (Kolesnick, 1987). Another DG analogue OaG gave a similar result which phorbol esters were unable to mimic. In addition, DiC_8 was able to stimulate sphingomyelin hydrolysis in PKC-downregulated cells. The authors concluded that this was a PKC-independent event. However, there are reports showing TPA-stimulated sphingomyelin synthesis in GH_3 cells (Kolesnick, 1898) and HL60 cells (Kiss *et al.*, 1988). Stimulation of sphingomyelin synthesis would mean a dilution of labeled sphingomyelin, thereby decreasing its specific radioactivity. A subsequent hydrolysis in the presence of TPA may not, therefore, be readily detected. Clearly, further detailed studies are needed before a valid conclusion regarding the apparently different effects of DiC_8 and TPA on sphingomyelin hydrolysis can be made.

1.6.3. *Involvement of Calcium in TPA- and DG-mediated Responses*

OaG and TPA have been shown to induce superoxide production by neutrophils, a characteristic of neutrophil activation. Low concentrations of OaG were shown to synergize with Ca^{2+} but Ca^{2+} had no effect on the TPA response (Ozaki *et al.*, 1986), and another study showed that high concentrations of OaG did not synergize with Ca^{2+} to activate neutrophils (Fugita *et al.*, 1984). This is entirely consistent with PKC activation, where low levels of DG synergize with Ca^{2+} to activate PKC but high levels are apparently sufficient in themselves to activate PKC. It is generally believed that TPA-mediated PKC activation in cells is independent of Ca^{2+}; OaG and TPA have been reported to activate platelets without causing a rise in intracellular calcium (Rink *et al.*, 1983). This observation is questionable because there is evidence that OaG and TPA can cause increases in intracellular Ca^{2+} in platelets (Ware *et al.*, 1985; Brass and Laposata, 1987). These discrepancies appear to be due to the

differencies in the agent used by various investigators to detect changes in intracellular Ca^{2+}. It is suggested that the more commonly used methods of detecting small localized changes in intracellular Ca^{2+} may not be sensitive enough (Johnson *et al.*, 1985; Ware *et al.*, 1985).

1.6.4. *Differential Biological Effects of TPA and other DG Analogues*

TPA and DG analogues such as DiC_8 and OaG have been suggested to act via different mechanisms in activating platelets and neutrophils, or on the maturation of HL60 cells. TPA and OaG caused platelet aggregation, a characteristic of platelet activation, and phosphorylation of a 40 kDa protein (Ashby *et al.*, 1985). In the same study low concentrations of OaG stimulated cAMP production whereas high concentrations of OaG did not. TPA was shown to inhibit forskolin-induced cAMP production. Therefore, high concentrations of OaG acted in a similar fashion to TPA. TPA generally causes sustained DG production. Since OaG is known to be metabolically unstable in cell culture (see beginning of section 1.6), a *transient* activation of PKC may have caused the increase in cAMP. TPA causes an irreversible activation of PKC and therefore, a comparison between effects mediated by low concentrations of OaG and TPA is not valid.

It is possible that certain metabolic products of OaG may mediate the effects normally attributed to the DG analogue itself. OaG may activate neutrophils due to its conversion to 1-oleyl-2-acetyl-phosphatidic acid (OaPA), a molecule structurally similar to PAF, a known activator of neutrophils (Ingraham *et al.*, 1982). Indeed, not only was exogenous OaPA more effective in releasing Ca^{2+} from the platelet dense tubular system than OaG itself (Laposata, 1987), but OaG was shown to be converted to OaPA in platelets (Kaibuchi *et al.*, 1983).

Another apparently different effect of OaG versus TPA frequently cited in the literature as an example of PKC-independent effects of OaG, is that of HL60 cell maturation. TPA has been shown to induce maturation of HL60 cells, which OaG was unable to mimic, although both agents activated PKC and stimulated PC biosynthesis (Kreutter *et al.*, 1985). In this study TPA was shown to cause the phosphorylation of 14 proteins, only 9 of whose phosphorylation was enhanced by OaG. OaG was unable to induce maturation of HL60 cells even when treating cells with fresh OaG every 2 h for 12 h. The authors concluded that PKC activation was necessary but insufficient for induction of maturation. It is possible that PKC-downregulation (or inactivation of PKC) is required for the maturation process. DG analogues such as DiC_8 and OaG are unable to downregulate PKC in MCF-7 (Issandou *et al.*, 1988) or in Swiss 3T3 cells (Issandou and Rozengurt, 1989). Therefore, the differences in the ability of TPA and other DG analogues to induce HL60 maturation may be due to the relative metabolic stabilities of these agents rather than activation of different pathways.

TPA and DiC_8 differ in their abilities to inhibit growth of MCF-7 cells, although both showed similar effects on cell morphology and the phosphorylation of a 28 kDa protein (Issandou *et al.*, 1988). In this study TPA induced the phosphorylation of more proteins than DiC_8. In addition, DiC_8 was unable to mimic TPA-induced PKC downregulation despite repeated additions to the culture medium. It, therefore, appears that DiC_8 was unable to mimic TPA-induced inhibition of proliferation due to its inability to downregulate PKC, rather than acting via a divergent pathway.

It is clear that most of the biological effects mediated by TPA and other DG analogues that are attributed to divergent pathways may be explained on the basis of their relative metabolic stabilities and consequently by their

abilities to activate or downregulate PKC. Indeed, recently Molina and Ashendel (1991) have shown by immunochemical techniques that DiC_8 induces the phosphorylation of PKC only half as much as TPA. Ohno *et al.* (1990) have demonstrated that autophosphorylation may be a necessary prelude to downregulation which may explain why DiC_8 was unable to cause downregulation of PKC in the studies cited in this thesis. Therefore, the poor efficacy of DiC_8 in inducing long-term responses may result in part from its very rapid metabolism and in part from its inability to downregulate PKC. A reevaluation of many of the so-called PKC-independent pathways is in order.

However, there *are* instances where this explanation may not suffice. Friedman *et al.* (1990) showed that OaG but not TPA enhanced melanogenesis in UV-irradiated melanocytes, and that downregulation of PKC by TPA augmented the response mediated by OaG. OaG but not TPA was able to inhibit forskolin-induced cAMP production in granulosa cells (Shinohara *et al.*, 1985), although both OaG and TPA were able to inhibit the maturation process.

1.7. THE CENTRALITY OF DG IN PC METABOLISM

It is evident from the previous sections that DGs can evoke potent effects on PC metabolism. DG can exert a regulatory influence on the PC biosynthetic pathway, not only by virtue of being a substrate for the cholinephosphotransferase (CPT) reaction but by regulating the subcellular distribution of the regulatory enzyme in the pathway itself. The regulatory effect of DG as a substrate has been shown by Jamil *et al.* (1992) where decreased levels of DG caused a decrease in PC biosynthesis. DG as a regulator of CT is evident in a report by Utal *et al.* (1991). It is also clear that the production of DG as a consequence of PC hydrolysis triggers PC biosynthesis and would appear to do this first by activating the pathway (via CT activation)

and then by itself acting as substrate because of its increased requirement by CPT. In this fashion, DG can also regulate its own levels.

The importance of DG in PC metabolism, therefore, must not be underestimated. It occupies a central position in PC metabolism by regulating PC biosynthesis at two levels and at the same time imposing a self-correcting mechanism on the pathway by regulating its own accumulation.

1.8. IS DG A SECOND MESSENGER?

There is mounting evidence in the literature that a phospholipase D is activated via PKC activation (upon hormone- or TPA-treatment of cells) to produce phosphatidic acid (PA) which is dephosphorylated by the action of the enzyme phosphatidate phosphohydrolase (PAP), giving rise to the so-called second phase of sustained DG production. The first phase of DG production is rapid and transient, is derived from PIP_2 hydrolysis and activates PKC, whereas the second phase is a consequence of PKC activation, is sustained and derived from PC hydrolysis (Exton, 1990; Billah and Anthes, 1990; van Blitterswijk *et al.*, 1991a and 1991b).

For a molecule to be an appropriate second messenger, it should be characteristically potent and short-lived. While phase 1 of DG accumulation clearly satisfies these characteristics, phase 2 of DG accumulation apparently does not. Therefore, the question arises: Is phase 2 DG a second messenger at least in those cells where it arises via PAP? The PA produced by phospholipase D appears to be a potent and transient signal, making it a suitable messenger. PA itself has been shown to stimulate potently DNA synthesis in cultured human mesangial cells (Knauss *et al.*, 1990) and protein phosphorylation in cell free systems (Bocckino *et al.*, 1991). PA may also be metabolized to lysophosphatidic acid (lysoPA) which stimulated lipid turnover and

mitogenesis in fibroblast cell lines (van Corven *et al.*, 1989). Indeed, it was later shown from the same laboratory that lysoPA but not PA was stimulatory for fibroblasts (Jalink *et al.*, 1990). This calls into question the status of the phase 2 of DG production. What is the biological significance of this DG - is it a second messenger or merely a by-product of PA metabolism and which takes care of housekeeping functions such as restoring PC homeostasis in activated cells? Indeed, it has been suggested by Martinson *et al.* (1990) that the conversion of PA to DG by PAP might be a 'turn-off' mechanism rather than an activation.

In the same context, LPC which is normally present at low concentrations within the cell has recently been shown to synergize with DG and Ca^{2+} to activate PKC (Asaoka *et al.*, 1991). LPC is normally rapidly cleared by its acylation to PC. Indeed LPC has been used to raise PC levels in hepatocytes (Jamil *et al.*, 1990). High levels of LPC would be potentially toxic to cells due to the detergent-like properties of LPC and, therefore, requires rapid clearance. Its transient state may make it a suitable second messenger.

It is not clear whether the second phase DG can target the activation of other proteins; the work presented in this thesis has identified CT as one target.

1.9. THE TERM 'SECOND MESSENGER' MAY BE IMPRECISE

As the cascade of biochemical events leading to various metabolic changes and/or DNA synthesis after cell activation is unravelled, more molecules mediating these events will be discovered. Consequently, it will be inappropriate to label all the molecules 'second messengers', as a second messenger for one event may well be a third or fourth messenger for the same event in another system. For example, PA or DG would be second messengers

in TPA-treated cells, whereas in hormone-treated cells PC hydrolysis to produce PA or DG is generally considered to be downstream of recognition by receptors, PIP_2 hydrolysis, Ca^{2+} mobilization and PKC activation (Exton, 1990). Stimulation of cells by certain hormones results in the production of cAMP due to activation of adenylate cyclase, or DG and inositol phosphates due to the activation of the PIP_2-specific phospholipase C. Inositol phosphates in turn cause the release of Ca^{2+} from intracellular stores. DG causes the activation of PKC which stimulates a PC-specific phospholipase D or phospholipase C to generate the second phase of DG production. Subsequent events leading to changes in nuclear transcription are largely unchartered.

The second messenger concept arose from E.W. Sutherland's observation that epinephrine activated adenylate cyclase to catalyze the production of cAMP on the inside of avian erythrocyte cell membranes (Sutherland *et al.*, 1965). The authors proposed a multimessenger system in which a first messenger (such as a hormone) interacts with sites at the cell membrane to stimulate formation of a second messenger which may modify enzyme activity. The second messenger may in turn stimulate the formation of a third messenger. A second messenger has been defined by G. Zubay in *Biochemistry* (second edition, Macmillan Publishing Company, New York) as 'a diffusible small molecule, such as cAMP, that is formed at the inner surface of the plasma membrane in response to a hormonal signal'. The discovery of other intracellular mediators effecting hormone- or growth factor-induced metabolic changes renders the current definition obsolete. According to the current definition, first and second phase DGs, inositol phosphates, PA, cAMP are all second messengers, although the exact sites of their production have not been uneqivocally identified. Ca^{2+} is also referred in the literature as a second messenger, although it is not clear from which intracellular site it

arises. Moreover, the term 'second messenger' implies a hierarchical status that may not be appropriate (inositol phosphates and Ca^{2+} are both termed as second messengers, yet one preceeds the other as is the case for the phases of DG production). Indeed, J.K. Grant cautioned that the term 'messenger' be used with caution (Sutherland *et al.*, 1965). Therefore, I propose that the term 'second messenger' be replaced by ARIEL (A Rapid Intracellular Effector Link) to embrace all such molecules that are presently called second messengers and those that are yet to be discovered. Since Ariel was an airy spirit, who frequently acted as a messenger in William Shakespeare's *The Tempest*, this term is in keeping with the concept of 'second messenger' biochemical molecules. Therefore, ARIELS would be defined as 'small molecules such as DGs, inositol phosphates and calcium, generated in response to extracellular activating signals, and effecting various biochemical responses such as protein phosphorylation, which may affect cellular metabolism and may ultimately lead to nuclear transcription'.

In this thesis the term 'second messenger' has been used as currently understood in the literature, which is that a second messenger is a molecule generated in response to an extracellular agent.

1.10. RATIONALE AND OBJECTIVES OF THESIS

As is apparent from the literature review in this chapter, TPA stimulates PC metabolism in a variety of cell types, including HeLa cells. The stimulation of PC biosynthesis appears to be due to the activation of the CDP-choline pathway via the rate-limiting enzyme, CT whose regulation in turn is diverse and complex. Therefore, the major objective of this study was to elucidate the mechanism(s) whereby TPA caused the activation of CT. A model for TPA-elicited CT translocation in Hela cells was proposed (Fig. 5).

The objectives of this thesis were to determine

1) whether CT was translocated to membranes upon TPA-treatment of HeLa cells

2) whether the translocation of CT was due to a reversible phosphorylation mechanism

3) whether increased DG levels or decreased PC levels due to TPA-treatment modulated association of CT with membranes

4) the subcellular location of translocated CT by immunocytochemical techniques.

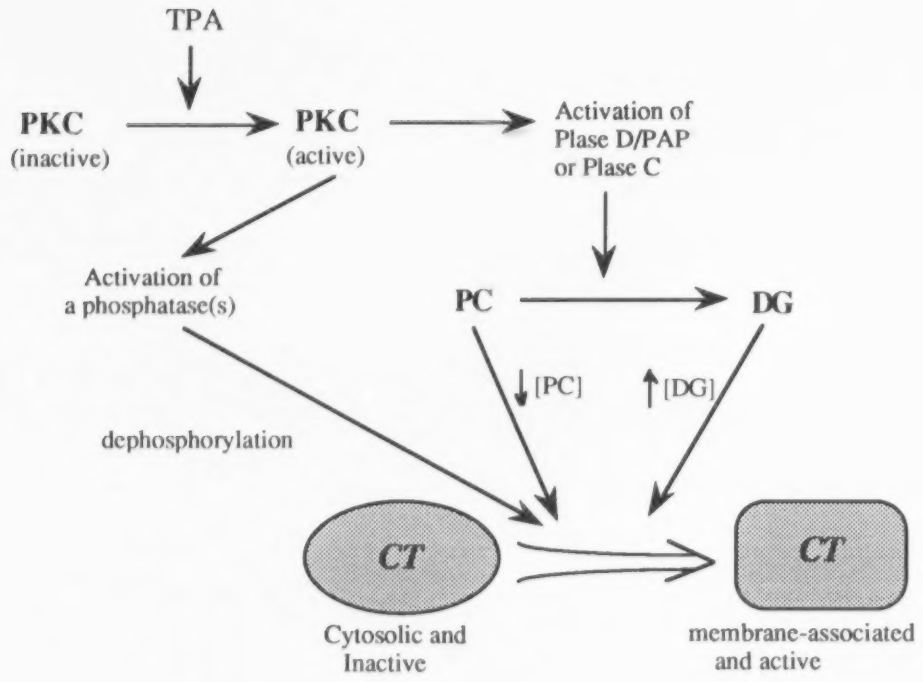

Fig. 5. Proposed Schematic Outline for CT Translocation to Membranes in TPA-Treated HeLa Cells. It was postulated that activation of PKC upon TPA treatment of HeLa cells caused (1) the activation of a phospholipase D (Plase D) followed by phosphatidate phosphohydrolase (PAP), or a phospholipase C (Plase C), which hydrolyzed PC to generate DG. The resulting decreased PC levels or increased DG levels would cause CT to translocate to the ER, or (2) activation of a phosphatase(s) which would dephosphorylate CT causing it to translocate either by making CT more hydrophobic, or causing other changes in the enzyme facilitating its interaction with membranes.

CHAPTER 2 EXPERIMENTAL PROCEDURES

2.1. Materials

All media and materials (including fetal bovine serum and antibiotics) for cell culturing were from Gibco, Canada. Tissue culture dishes were from Falcon. Solvents (methanol, ethanol, and diethyl ether) were from BDH and were of reagent grade. Hexane and acetonitrile were from T.J Baker Inc.

Radiochemicals, [γ-^{32}P]ATP (3000 Ci/mmol), ^{32}P-orthophosphate (carrier-free), [*methyl*-^{3}H]choline chloride (15 mCi/mmol), [2-^{3}H]glycerol and ^{35}S-methionine were from Amersham. ACS scintillation fluid and a monoclonal antibody to PKC (MC 5) were purchased from Amersham.

Nitrocellulose membranes, dye reagent for protein assays and molecular weight standards for SDS-PAGE were from Bio-Rad. The Immobilon-P membrane was from Millipore.

Glass and plastic-backed silica gel G 60 (20 x 20 cm) plates and reversed phase C_{18} (5 x 10 cm) plates for thin layer chromatography were purchased from BDH chemicals.

Reagents for the BCA protein assay method were from the Pierce Chemical Company.

The mixture of fatty acid standards, transmethylation reagents (BF_3-methanol, 10% w/w; and BF_3-butanol, 14% w/w) and the 10% DEGS column for GLC were from Supelco.

Octyl-β-D-glucoside and DG kinase inhibitor were from Boehringer Mannhiem. *Escherichia coli* DG kinase for DG mass measurements was from Lipidex Inc. Protein A Sepharose CL 4B used in immunoprecipitation studies was from Pharmacia LKB Biotechnology AB, Uppsala, Sweden.

Several reagents were generously shared by other scientists : 1,2-*sn*-dioctanoyl[2-^{3}H]glycerol and DG lipase inhibitor were provided by Dr. David Severson from the University of Calgary. Purified PKC used as a standard in PKC-immunoblotting was from Dr. Robert M. Bell (Duke University Medical Centre, North Carolina), U-73122, a phospholipase C inhibitor was provided by Dr. John Bleasdale (The Upjohn Company, Kalamazoo, MI), lovastatin and ^{125}I-protein A were obtained from the laboratories of Drs. Shinji Yokoyama and Wolfgang Schneider (University of Alberta), respectively. Reagents used in the immunofluorescence studies were obtained from the laboratory of Dr. Ann Acheson (University of Alberta).

2.2. Cell Culturing

2.2.1. *HeLa Cells*

HeLa cells (ATCC CCL2) were obtained from the American Type Culture Collection and maintained in growth medium Dulbecco's Modified Eagle's Medium buffered with 10 mM Hepes and supplemented with 50 U/ml penicillin, 50 U/ml streptomycin and 10% fetal bovine serum). Cells were maintained in 100 mm tissue culture dishes at 37°C, 5% CO_2 and 90% relative humidity. For passaging the adherent cells were first trypsinized with 0.1% trypsin in Hank's salt solution (minus Ca^{2+} and Mg^{2+}), pH 7.5, and resuspended in growth medium. The trypsinized cells were diluted five times before plating in tissue culture dishes. For experiments cells were plated in 60 mm tissue culture dishes at a density of 0.5 x 10^{6} cells/dish and used 2-3 days later.

2.2.2. *REF 52 Cells*

Ref 52 (REF A) cells were obtained from Dr. Myles Cabot and were maintained in a 3:1 mixture of Dulbecco's Modified Eagle's Medium : F-12

Nutrient Mixture (Ham's F-12) and buffered with 20 mM Hepes and 1.2 g/l sodium hydrogen carbonate. The media were supplemented with antibiotics (ampicillin, 0.024 mg/ml; penicillin, 0.12 mg/ml and streptomycin sulphate, 0.27 mg/ml), 50 μM α-aminoethanol, 0.005 nM sodium selenite and 0.4 g/l of histidine-HCl. The cells were trypsinized, passaged and plated for experiments as described above for HeLa cells.

2.3. Composition of Buffers

Bouin's Solution - 2% paraformaldehyde, 5% picric acid, 5% sucrose in 0.2 M phosphate buffer, pH 7.6; *Buffer A* - 50 mM Tris-HCl, pH 7.4, 0.15 M NaCl, 2 mM DTT, 1 mM EDTA, 0.1 mM PMSF; *Buffer R* - 10 mM Tris-HCl, pH 7.4, 250 mM sucrose, 1 mM EDTA and 0.1 mM PMSF; *Digitonin buffer* - 0.5 mg/ml digitonin, 10 mM Tris-HCl, pH 7.4, 0.25 M sucrose, 0.5 mM phenylmethylsulfonyl fluoride; *Hepes-buffered saline(HBS)* - 20 mM Hepes, pH 7.5, 0.15 NaCl; *RIPA buffer* - 50 mM Tris-HCl, pH 7.4, 0.15 M NaCl, 1% nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS; *Tris-buffered saline (TBS)* - 25 mM Tris-HCl, pH 7.4, 0.15 M NaCl; *TTBS* - TBS with 0.05% Tween-20; *Blotto* - TTBS with 5% Carnation non-fat skim milk powder (Carnation Inc., Toronto); *lysis buffer* - 25 mM Tris-HCl, pH 7.4, 0.25 M sucrose, 5 mM EGTA, 2 mM EDTA, 1 mM DTT, 10% glycerol, 0.5 mM phenylmethylsulfonyl fluoride, 4 μg/ml leupeptin; *Laemmli buffer* - 62.5 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 5% 2-mercaptoethanol (or 100 mM dithiothreitol) and 0.001% bromophenol blue.

2.4. Estimation of Protein

Since the measurement of protein from membrane fractions constituted a significant part of this study, it was important to make sure that the method utilized did not have serious interference due to the lipid constituent of

membranes. Three different methods of protein measurement were tried with bovine serum albumin[1] (BSA) as the standard against which actual samples were measured. Fig. 6 shows the standard curves obtained by utilizing the (*A*) Bio-Rad, (*B*) BCA and (*C*) Lowry methods.

(A) *the Bio-Rad method*

This method is based on the method of Bradford (1976) and utilizes the principle of the binding of the dye Coomassie Brilliant Blue G-250 to proteins. The interaction of the dye with proteins shifts the absorption maximum of the dye from 465 to 595 nm.

(B) *the BCA protein assay method* (Smith *et al.*, 1985)

The reagents for this method are commercially available from the Pierce Chemical Company. The method utilizes the biuret reaction in which Cu(I) ions are formed when proteins react with alkaline Cu(II) ions. The resulting Cu(I) ions are detected by a sensitive and selective reagent, bicinchoninic acid (BCA). The resulting complex between Cu(I) and BCA gives a purple color which absorbs strongly at 562 nm. According to Kessler and Fanestil (1986) large amounts of lipids in samples can give artificially high absorbancies in this method. However, this was not apparent when this method was utilized for measuring the protein content of microsomal samples in the present study presumably because the membrane lipids in the assay were not present to the extent used by Kessler and Fanestil (1986).

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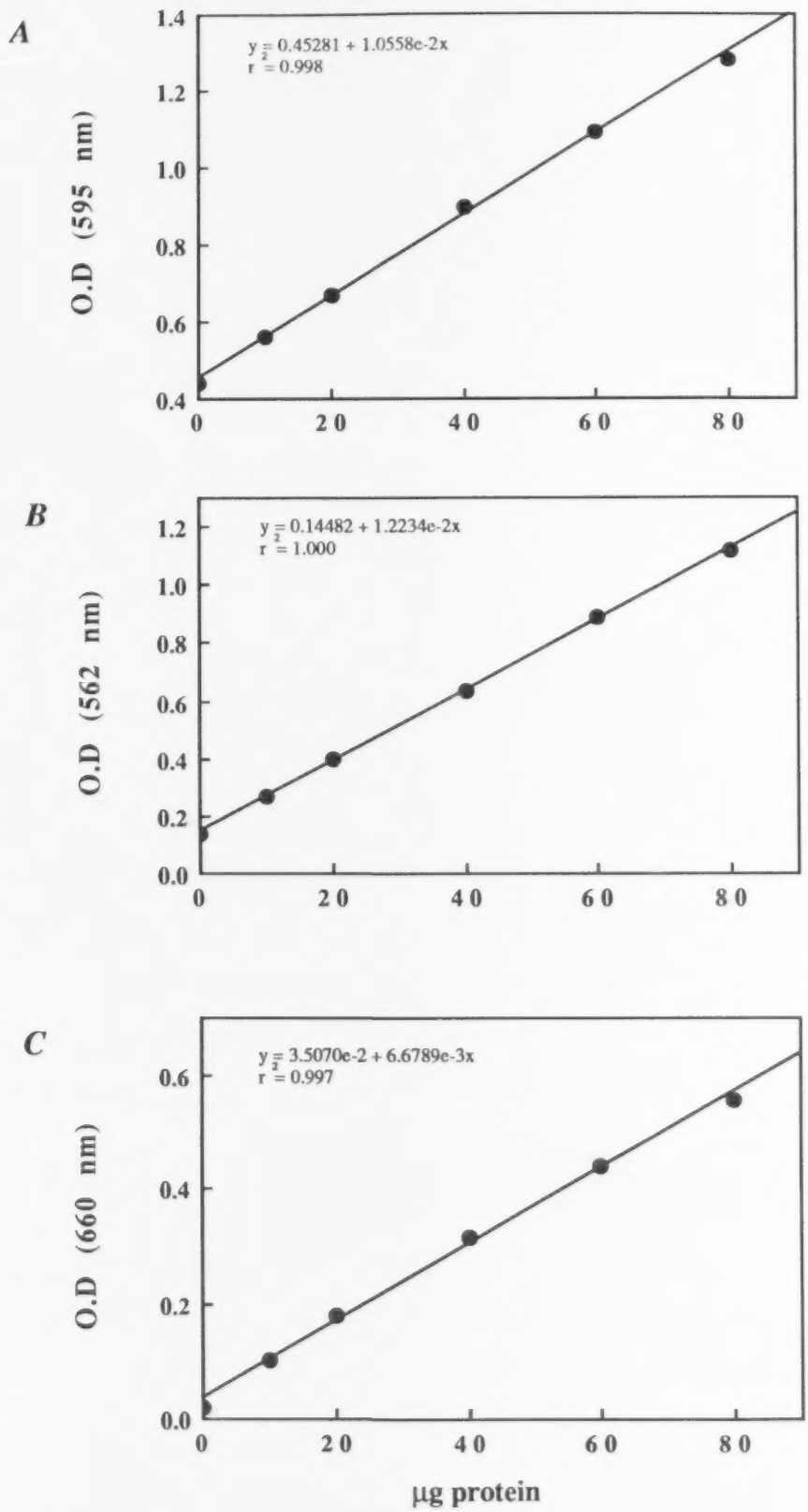
[1]BSA standard was prepared as a 1 mg/ml stock from fatty acid-free BSA and was quantified with an absorption of 0.66 at 280 nm.

Fig. 6. Standard Curves for the Measurement of Protein. 10 to 80 µg standards were prepared from a 1 mg /ml BSA stock to obtain the standard curves for protein measurement using the (*A*) Bio-Rad (*B*) BCA and (*C*) Lowry methods.


A
y = 0.45281 + 1.0558e-2x
r = 0.998
O.D (595 nm)
1.4
1.2
1.0
0.8
0.6
0.4
0
20
40
60
80
B
y = 0.14482 + 1.2234e-2x
r = 1.000
O.D (562 nm)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0
20
40
60
80
C
y = 3.5070e-2 + 6.6789e-3x
r = 0.997
O.D (660 nm)
0.6
0.4
0.2
0.0
0
20
40
60
80
µg protein

(C) *the Lowry Method* (Lowry *et al.*, 1951)

This is the most sensitive method, although laborious and inconvenient for the routine assay of a large number of samples. Moreover, a large number of biochemicals (which are frequently important constituents of buffers) cause interference in the assay.

The absolute values of cytosolic and microsomal samples varied with the three different methods. However, the differences between samples were constant among these methods. The Bio-Rad method was the method of choice in this study due to its convenience and economy and its relative resistance to various interfering biochemicals compared to the BCA method and especially the Lowry method.

2.5. Lipid Extractions

Lipid extractions were carried out by the method of Bligh and Dyer, 1959. Where specified 1 M NaCl (or 0.1 N HCl) instead of water was used in the separation of the phases.

2.6. Phosphorous Assay

Lipid phosphorous was determined by the method of Rouser *et al*, 1966. Sterile solutions of 1 mM KH_2PO_4 were used to generate standards against which samples were measured at 820 nm.

2.7. Pulse-Chase Experiments

HeLa cells in 60 mm dishes were prelabeled in 2 ml medium containing 2 μCi/ml [^{3}H]choline chloride for 1 h or 2 μCi/ml [2-^{3}H]glycerol for 24 h. The labeled medium was removed, cells were rinsed three times with warm medium and incubated in unlabeled medium in the absence or presence of agents as

specified in the Figure legends. Unless specified the various agents were dissolved in DMSO. Cells used as controls in the experiments in which these agents were used were incubated with 0.1% DMSO. This was usually the final concentration of DMSO when cells were treated with the agents. In experiments where two agents were used together the controls were incubated in the presence of 0.2% DMSO, the highest concentration of DMSO to which cells were subjected.

2.8. SDS-Polyacrylamide Gel Electrophoresis

Samples were prepared for SDS-PAGE by heating in an equivalent volume of Laemmli Buffer (62.5 mM Tris.HCl, pH 6.8, 2% SDS, 10% glycerol, 5% 2-mercaptoethanol and 0.001% bromophenol blue) for 10 min at 80°C.

SDS-PAGE was performed on 1.5 mm thick gels as described by Laemmli, 1970. The stacking gels were 3% and samples were separated on 10% separating gels containing 0.1% SDS. Electrophoresis was performed at a constant current (25 mA) for 6-7 h or 10 mA for approximately 15 h.

2.9. Electroblotting of Proteins

After SDS-PAGE, the proteins in the gels were transferred onto nitrocellulose (in the case of protein kinase C) and immobilon-P (in the case of CT) membranes in a transfer buffer system containing 25 mM Tris, 192 mM glycine and 20% methanol. In the case of PKC, the transfer was carried out at 200 mA for 6 h and in the case of CT, the transfer was at 200 mA overnight for 12-14 h (or 300 mA for 10 h).

2.10. Preparation of Oleate Stock

Oleate was prepared as a 35 mM stock by dissolving oleic acid in medium (DMEM) containing a 20% molar excess of potassium hydroxide. This stock was aliquoted and stored frozen at -20 °C. When required it was thawed and added directly to cells in growth medium.

2.11. Assay of CT

2.11.1. *Preparation of PC : Oleic Acid Vesicles*

PC : oleic acid (1 : 1 molar ratio) vesicles were prepared by mixing the appropriate volumes of stock solutions of PC and oleic acid in a round bottomed flask. The solvent was evaporated under a stream of nitrogen followed by evaporation under vacuum in a rotary evaporator. Buffer A (50 mM Tris.HCl, pH 7.4, 0.15 M NaCl, 2 mM DTT, 1 mM EDTA) was added to give a 10 mM stock, and sonicated with a microtip probe sonicator until a translucent solution was obtained. The stock solution was stored at 4 °C. When required the 10 mM stock was diluted to 2 mM with buffer A.

2.11.2. *Preparation of Phospho[methyl-^{3}H]choline*

The substrate for the CT assay was prepared by converting 2 mCi of [*methyl*-^{3}H]choline chloride (1 mCi/ml) after drying under nitrogen to phospho[*methyl*-^{3}H]choline with 0.25 units of dialyzed choline kinase. The reaction was carried out at 37 °C for 1 h in the presence of 0.1 M Tris.HCl, pH 8.0, 10 mM $MgCl_2$, and 10 mM ATP. The reaction was stopped by placing the tube in a boiling water bath for 2 min. The tube was centrifuged at 5,000 rpm for 5 min and the supernatant applied along the length of a glass-backed silica gel G-60 TLC plate (20 x 20 cm) 2 cm from the bottom of the plate. The plate was developed in methanol : 0.6% NaCl : ammonia (10 : 10 : 1) for 3 h. Lines 1 cm

apart were drawn horizontally across the plate. 1 x 1 cm square sections were marked vertically along the middle of the plate and scraped into tubes containing 2 ml of water. After vortexing 20 µl aliquots were removed for scintillation counting. The lane where the radioactivity peaked was scraped and the silica extracted with 2 ml of water. Extraction with water was repeated until most of the radioactivity was eluted from the silica. The washes were pooled and non-radioactive ('cold') phosphocholine was added so that the final concentration was 15 mM and the specific radioactivity was 9-10 µCi/µmol.

2.11.3. *The Assay Procedure*

CT assays were performed essentially as described by Weinhold *et al.* (1986) with modifications (Yao *et al.*, 1990). Briefly, 60 µl samples were added to tubes containing 10 µl of 2 mM PC : oleate vesicles. 30 µl of the assay buffer containing 1.5 mM phospho[*methyl*-^{3}H]choline as substrate, 7.5 mM magnesium acetate, 75 mM Tris-HCl, pH 6.5 and 2 mM CTP, was added to each tube and incubated with shaking at 37 °C. After 30 min (unless specified), the tubes were removed and placed in a water bath at 85 °C for 2 min to stop the reaction. The tubes were centrifuged at 1000 x g for 5 min and 15 µl of the supernatant was spotted on plastic-backed silica gel G-60 TLC plates (10 x 20 cm) alongwith 5 µl of a mixture of standards containing 80 mg/ml of phosphocholine and 15 mg/ml of CDP-choline. The metabolites were separated using the following solvent system : methanol : 0.6% NaCl : ammonium hydroxide (10 : 10 : 1, v/v) The plates were sprayed lightly with 0.1% 2',7'-dichlorofluorescein in methanol. The CDP-choline was viualized under U/V light and scraped into plastic scintillation vials containing 0.5 ml water. 5 ml of ACS scintillation fluid was added. The vials were counted the next day after vortexing. CT activity was expressed as nmol CDP-choline formed min^{-1}.

2.12. Assay of Lactate Dehydrogenase (LDH)

LDH assays were carried out essentially as described by Bergmeyer and Bernt (1974), with modifications. Briefly, 10 to 20 µl of the digitonin-released cytosol (in triplicate) was placed in a 96 - well microtitre plate. 200 µl of a mixture containing 175 mM Tris-HCl, pH 7.4, 0.16 mg/ml sodium pyruvate and 0.24 mg/ml NADH was added to the sample, and the decrease in absorbance monitered every 30 sec. using a multi-well plate reader EAR 340 AT (SLT - labinstruments, Austria). The LDH activity was calculated from the decrease in absorbance at 340 nm per min and a molar extinction coefficient of 6.22×10^3.

2.13. Cell Disruption Methods

2.13.1. *Dounce Homogenization*

Cells in 0.5 ml of Buffer A were homogenized in a tight fitting glass homogenizer with 40 strokes of a motor-driven teflon pestle. By this method approximately 90% of the cells were broken and mostly cell debris was visible under the light microscope in the presence of Trypan Blue.

2.13.2. *Nitrogen Cavitation*

Cells in 0.5 ml of Buffer A were placed in the steel chamber of a mini-bomb apparatus which had been equilibrated on ice for 15 min. Nitrogen gas was applied at 500 psi for 5 min. The gas supply was turned off and the resulting homogenate was slowly released through a narrow outlet from the chamber. Visualization of the homogenate by light microscopy revealed only cell debris and no intact cells.

2.13.3. *Digitonin Permeabilization of Cells*

This was performed essentially as described previously (11). Cells in 60 mm dishes were rinsed twice with cold PBS after various treatments, and permeabilized with 1 ml digitonin buffer for 5 min (except where specified otherwise) at 0°C with occassional gentle shaking. The released cytosolic contents were removed into Eppendorf tubes which were centrifuged at 13,000 x g in a microfuge for 2 min to pellet any cell debris or particles. The supernatants (cytosolic fractions) were transferred to clean tubes.

The cell ghosts remaining on the dishes after digitonin treatment were rinsed once with PBS, and scraped into 0.4 ml of Buffer R (10 mM Tris-HCl, pH 7.4, 0.25 M sucrose, 1 mM EDTA and 0.1 mM phenylmethylsulfonyl fluoride). The scraped cell ghosts were sonicated with two 10 second bursts at 30% power output with a sonicator equipped with a microtip probe (model W-385, Heat Systems Ultrasonics Inc.) and termed the particulate fraction.

2.14. Preparation of Microsomes

Microsomes were prepared from cells as described by Cornell and Vance (1987). Briefly, after rinsing with cold PBS, cells from 100 mm dishes were scraped into 1 ml of Buffer A (10 mM Tris-HCl, pH 7.4, 0.15 M NaCl, 10 mM NaF, 0.1 mM PMSF, 1 mM EDTA) and homogenized with 40 strokes of a tight-fitting Dounce homogenizer. The homogenate was centrifuged at 10,500 x g for 10 min in a JA 20 rotor using a Beckman Model J2-21 centrifuge to sediment mitochondria, nuclei, cell debris and unbroken cells. The so-called post-mitochondrial fraction supernatant was centrifuged at 4°C for 1 h at 43,000 rpm (100,000 x g) in a Ti 70.1 rotor using an L8-M ultracentrifuge. In some experiments the post-mitochondrial fraction was centrifuged at 4°C for 15 min at 99,000 rpm (350,000 x g) in a TL 100 Beckman ultracentrifuge using a TL

100.2 rotor. The resulting supernatant was saved as the cytosol and the pellet was re-suspended in 0.5 ml of Buffer R (10 mM Tris-HCl, pH 7.4, 0.25 M sucrose, 1 mM EDTA and 0.1 mM PMSF) to obtain the microsomal fraction.

Homogenates obtained from nitrogen cavitation experiments were fractionated into cytosol and microsomes as described above.

2.15. Quantitation of Diacylglycerol Mass

Two methods are described here, the first one was developed from a commercially available kit, and the second one was adopted as the method of choice for routine DG mass determination after DG kinase became commercially available in a purified form.

2.15.1. *Measurement of DG Using a Colorimetric Assay*

This method was modified from a triglyceride assay kit purchased from *Wako Pure Chemical Industries, Ltd.* (Osaka, Japan). It is based on the enzymatic hydrolysis of triglycerides (in this case DG) with the subsequent determination of liberated glycerol by colorimetry (see Appendix A). Bucolo and David (1973) first published the method using lipase to generate free glycerol with its subsequent measurement using glycerol kinase. Diacylglycerol is acted upon by lipoprotein lipase (LPL) to glycerol and free fatty acids. The glycerol thus produced is converted to glycerol-3-phosphate by glycerol kinase (GK) in the presence of ATP. This glycerol-3-phosphate is then oxidized to glycerol-3-phosphate oxidase (GPO) to yield hydrogen peroxide, which yields a red colour compound upon oxidative condensation with *p*-chlorophenol and 4-aminoantipyrine in the presence of peroxidase. The amount of DG in the original sample is then determined by measuring the

absorption of the developed colour at 505 nm as compared with that of a set of standards.

The colour reagent was prepared by reconstituting 2 mg of a mixture containing 29 units of LPL, 1.8 units of GK, 3.6 units of GPO, 1.7 units of peroxidase, 0.9 mg of ATP and 0.1 mg of 4-aminoantipyridine in 1 ml of buffer containing 0.05 M Tris-HCl pH 7.5 with 0.07% *p*-chlorophenol.

a) Standard Curve for Dipalmitin - Dipalmitin standards were prepared from a 1 mg/ml stock; 1, 2, 4, 8, 10 µg in chloroform and dried under nitrogen. The standards were resolubilized in 15 µl of 2-propanol. 300 µl colour reagent was added to each tube and the tubes shaken for 30 min at room temperature. The tubes were then centrifuged at 14,000 x g briefly to pellet any particles. A volume of 200 µl was transferred to a 96-well microtitre plate and the absorbance measured at 492 nm using a multi-well plate reader EAR 340 AT (SLT-labinstruments, Austria). A typical standard curve is represented in Fig. 7.

b) Preparation of samples - One, 100 mm, dish was used for one sample. The cells were scraped into 0.7 ml PBS and sonicated for 10 sec x 2. 0.5 ml of the homogenate was removed for lipid extraction and an aliquot of the remainder for protein estimation. At this point 15 µl of ^{3}H-labelled DG tracer (see below) was added to the sample. The DG in the lipid fraction was separated by thin layer chromatography using diethyl ether : hexane : acetic acid (30 : 70 : 2). The DG bands were visualized with iodine which was let to vapourize overnight under vacuum. The DG bands were scraped and the DG eluted from the silica with 0.5 ml of diethyl ether 3 times. The washes were pooled and dried under nitrogen. 15 µl of 2-propanol was added to resolubilize the DG

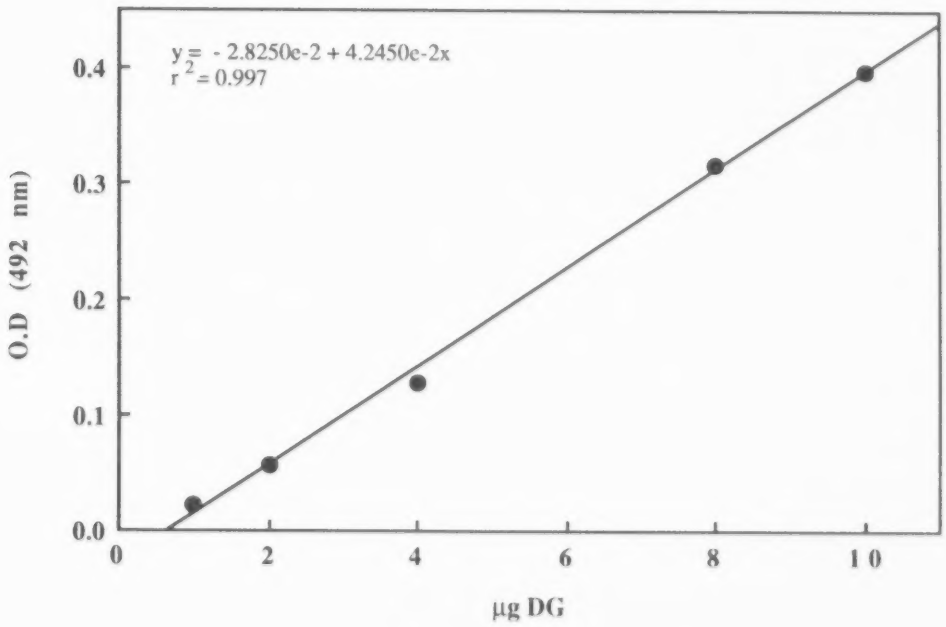


Fig. 7. Standard Curve for the Measurement of Dipalmitin. Increasing amounts of dipalmitin were subjected to a colorimetric analysis using the Wako Kit. The resulting color was measured at 492 nm.

followed by 300 µl of colour reagent. A set of dipalmitin standards was also measured each time to obtain a fresh standard curve against which the sample absorbancies were quantitated. Aliquots were then counted by liquid scintillation and the % recovery calculated. Generally, the recovery was 60 - 70%. The sample values were corrected for recovery and expressed as nmol DG per mg protein (assuming MW of DG to be 540).

This method, apart from being lengthy and laborious, has serious draw backs; the sensitivity was only to 2 µg i.e., 4 nmol DG, and large amounts of sample were required. Moreover, the results were inconsistent and the absolute values varied widely from experiment to experiment. However, the method did yield expected results of DG accumulation upon treatment of cells with TPA.

c) Preparation of ^{3}H-labelled DG for use as tracer in DG mass measurements - 4, 100 mm, dishes of HeLa cells were incubated with medium containing 6 µCi [2-^{3}H]glycerol per dish for 2.5 h. By this time the radiolabeling of the DG is at a steady state (see section 3.2.1). Cells from the 4 dishes were pooled and lipids extracted. Unlabelled cells from 4 dishes (2 dishes were pooled for one sample) were treated similarly. DG was separated by TLC, visualized, and eluted as described above. The two unlabelled samples were resolubilized with 15 µl of 2-propanol and proceeded with DG quantitation as described above. 5.5 µg DG was obtained from 2 pooled, 100 mm, dishes of HeLa cells.

The DG obtained from the labeled cells was dried under nitrogen and redissolved in 2 ml chloroform. Aliquots of 10 and 20 µl were dried and subjected to liquid scintillation counting. The specific radioactivity was calculated to be 14,400 dpm/µg DG. 15 µl (i.e., 0.11 µg) was used as a tracer in measurements of DG mass.

2.15.2. *Measurement of DG Mass Using the DG Kinase Method*

This method for measuring DG has several advantages over the previous one:

i. There are relatively fewer steps in the protocol and can be performed on a routine basis

ii. It is very sensitive and can detect DG levels as low as 100 pmol i.e., it is more than ten times as sensitive as the previous method.

The method relies on the quantitative conversion of DG to phosphatidic acid by *Escherichia coli* 1,2-*sn*-diacylglycerol kinase (Lipidex Inc.) in the presence of [γ-^{32}P]ATP. The method was first described by Priess *et al.* (1986) and later modified by Wright *et al.* (1988). The modified method was used in this study. After lipids were extracted from the respective samples, aliquots of the chloroform phase were dried under nitrogen and resolubilized with 20 µl octyl-β-D-glucoside/cardiolipin (7.5% octyl-β-D-glucoside, 5 mM cardiolipin in 1 mM diethylenetriaminepentaacetic acid [DETAPAC]) by alternatively sonicating in a Branson 1200 bath sonicator and vortexing. To these samples were added 80 µl aliquots of the following assay mixture. For each 5 µl of DG kinase containing 10 milliunits, there were 50 µl of 2x assay buffer (100 mM imidazole.HCl, pH 6.6, 100 mM NaCl, 25 mM $MgCl_2$, 2 mM EGTA), 10 µl of 20 mM freshly prepared dithiothreitol, 10 µl of [γ-^{32}P]ATP[2] (specific activity = 10^5 to 5 x 10^5 cpm/nmol) and 5 µl water. This mixture was heated at 30°C for 25 min before 80 µl aliquots were added to the tubes containing the samples. The assay tubes containing the assay mixture were incubated for 25 min at 30°C with vigorous shaking. The reaction was terminated by adding 1.88 ml C : M (1 : 2). Lipids were extracted essentially by the method of Bligh and Dyer except that 1

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[2]Carrier ATP was quantified by absorbance at 259 nm using a millimolar extinction coefficient of 15.4.

M NaCl was used instead of water. The organic phase was washed twice with 2 ml of 1% perchloric acid. Aliquots of the organic phase were spotted on 20 x 20 cm glass-backed silica gel 60 plates for TLC along with PA standard and the plates were developed in chloroform : acetone : methanol : acetic acid : water (10 : 4 : 3 : 2 : 1). Upon autoradiography of the plate three phosphorylated spots were seen, the major one corresponding to that of PA. This solvent system separated the labelled PA (R_F = 0.6) from ceramide phosphate (R_F = 0.4 - 0.45). In addition there was an unidentified band exhibiting an R_F value of 0.25. This unidentified spot may be lysophosphatidic acid because the DG kinase used in this assay is known to phosphorylate monoglyceride to this lipid (Bohenberger and Sanderman, 1982). Routinely, the PA bands were visualized with iodine and scraped into plastic scintillation vials containing 0.5 ml water. 5 ml scintillation fluid was added and the vials counted in a liquid scintillation counter (Beckman LS 3801).

Since the amount of carrier ATP added per sample was known, the specific activity of ATP was determined by counting 10 µl of the assay mixture added to the samples.

This assay was also used to quantify DiC_8 levels. The dioctanoylphosphatidic acid formed comigrated with ceramide phosphate as visualized by autoradiography. Therefore, the values obtained for DiC_8 are expressed after subtracting control values.

a) Preparation of DG standard by phospholipase C digestion of PC - This procedure was carried out essentially as described by Kuksis *et al.* (1981). 100 mg egg PC was dissolved in 1 ml diethyl ether. 4.1 ml of 17.5 mM Tris-HCl, pH 7.3, 1 ml of 1% $CaCl_2$ and 1 unit of *Clostridium welchii* phospholipase C was added. The reaction was carried out at 30°C for 3 h with vigorous shaking. DG was extracted by adding 200 µl water and 6.5 ml hexane. The upper hexane

phase was transferred to a clean pre-weighed glass tube and dried under nitrogen. The tube was reweighed and 81 mg of DG was obtained i.e. 130 µmol DG (let M.W. of DG = 620.97). The DG was dissolved in a known amount of hexane and aliquots containing 2 nmol DG were put in clean tubes, dried under nitrogen and stored at -20°C until required.

b) Standard curve for DG mass - Fig. 8 shows a typical standard curve obtained after assaying a set of DG standards prepared as described above (the use of commercially available DG was avoided because of the presence of significant amounts of 1,3-DG).

2.16. Antibodies to CT

Antibodies were raised against a peptide sequence within CT and affinity purified by Dr. Haris Jamil in this laoratory. The procedure is described in a manuscript in Press (Jamil *et al.*, 1992). Briefly, a synthetic peptide frgment of CT between amino acid residues 164 and 176 (asp-phe-val-ala-his-asp-asp-ile-pro-tyr-ser-ser-ala) according to Kalmar *et al.* (1990) conjugated with bovine serum albumin and keyhole limpet hemocyanin (KLH) was purchased from the Alberta Peptide Institute, University of Alberta, Edmonton. Rabbit antibodies were raised to the peptide-KLH conjugate and purified initially on a Protein A Sephrose CL4B column. The eluted IgG was subsequently affinity purified on a column containing synthetic peptide-BSA conjugated to CNBr-activated Sepharose 4 B. The unadsorbed IgG was saved and used in CT immunoprecipitation and immunofluorescence studies.

2.17. Immunoblotting of CT

Cells were grown to confluency in 100 mm dishes. Four dishes that had

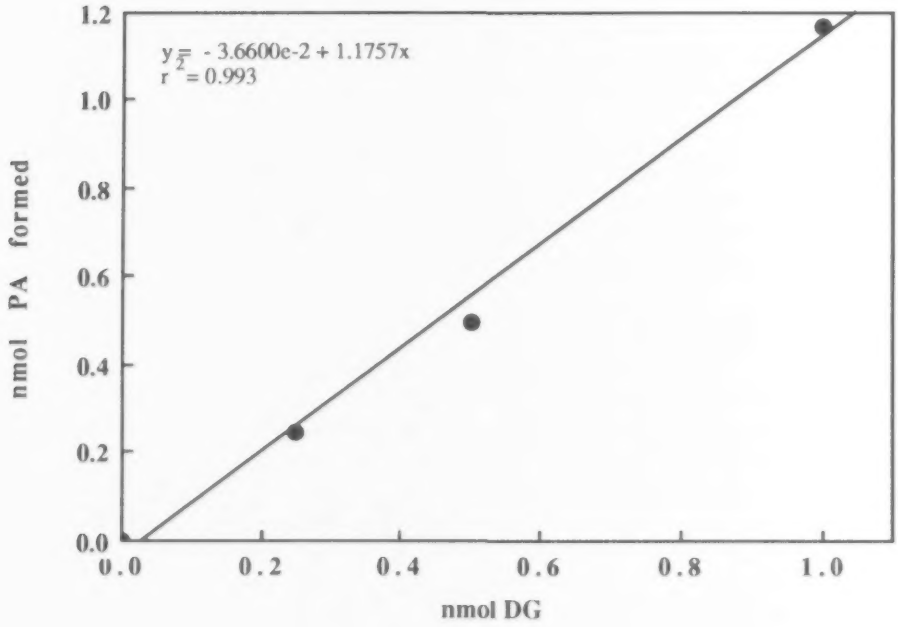

Fig. 8. Standard Curve for the Measurement of DG Mass.

Increasing amounts of DG were converted to phosphatidic acid (PA) in the presence of [γ-^{32}P]ATP using DG kinase. The products were separated by TLC and the area corresponding to PA was scraped and subjected to liquid scintillation counting.

been incubated in the absence or presence of 100 nM TPA for 1 h were digitonin-permeabilized with 2.5 ml digitonin buffer per dish for 5 min. The released cytosol was retained. The cell ghosts remaining on the dishes were rinsed once with PBS, scraped into PBS and centrifuged at 100,000 x g for 1 h in a Ti 70.1 rotor in a Beckman L8-M ultracentrifuge at 4°C. The supernatant was discarded and the pellet was resuspended in 0.3 ml RIPA buffer and sonicated with 2 bursts of 10 s each. This was allowed to sit on ice for 15 min with occasional vortexing and spun at 350,000 x g for 15 min at 4°C in a TL 100.2 rotor using a Beckman TL 100 ultracentrifuge. The supernatant was saved as the particulate fraction.

Specific amounts of cytosolic and particulate fractions were prepared for SDS-PAGE by heating the fractions in Laemmli buffer at 80°C for 10 min. The proteins in the samples were separated by SDS-PAGE (10% polyacrylamide gel), transfered to an Immobilon-P membrane using a transfer buffer containing 25 mM Tris, 192 mM glycine, 20% methanol for 10 h at 0.3 A. Unoccupied sites on the membrane were blocked by incubating in Blotto for 4 h, before incubating overnight with gentle rocking in Blotto containing a rabbit polyclonal antibody to CT at a 1:500 dilution at 4°C. The antibody solution was removed and the membrane rinsed 4 times with Blotto (10 min /rinse).

2.17.1. *Visualization of CT Using ^{125}I-Protein A*

After incubating the blotted membrane with anti-CT antibody followed by the necessary rinses, the membrane was incubated in blotto containing ^{125}I-Protein A (1.0 to 0.5 x 10^6 cpm/ml) for 1 h. The radiolabeled blotto was removed and the membrane rinsed 2 times with blotto (5 min/rinse) and 2

times with TTBS. Subsequently the membrane was air-dried and exposed to X-ray film at -70°C in a tight-fitting film cassette with intensifier screens.

2.17.2. *Visualization of CT Using Chemiluminescence*

Following incubation of the blotted membrane with the primary antibody and necessary rinses, the membrane was incubated in Blotto containing goat anti-rabbit immunoglobulin conjugated to horse radish peroxidase (1:2000 dilution) for 1 h, then rinsed twice with Blotto (5 min/rinse), twice with TTBS (5 min/rinse) and finally twice with TBS (10 min/rinse). The membrane was exposed to ECL detection reagents (Amersham Corp.) for 2 min. The ECL was detected by exposure of the membrane (sealed in a polyester pouch) to X-ray film for 30 min (the principle of the detection is illustrated in Appendix B). The sensitivity of this method enabled the visualization of membrane-associated CT which is present at low levels.

2.18. Immunoprecipitation of CT

2.18.1. *Titration of CT From HeLa Cell Cytosol With Antibody to CT*

Cytosol was obtained as usual by digitonin-permeabilization of HeLa cells. To 100 µl aliquots were added increasing amounts of affinity purified α-CT IgG (0 to 10 µg). The final volume was made to 200 µl with PBS, and the tubes were shaken for 1 h at 4°C. 50 µl of Protein A-Sepharose CL-4B (0.125 g/ml in PBS) was added to each tube and shaken again for 1 h at 4°C. The tubes were centrifuged in a microfuge at 14,000 x g for 1 min. 60 µl of the resulting supernatant was removed to assay for CT. The decreased activity in the supernatant after immunoprecipitation is illustrated in Fig. 9.

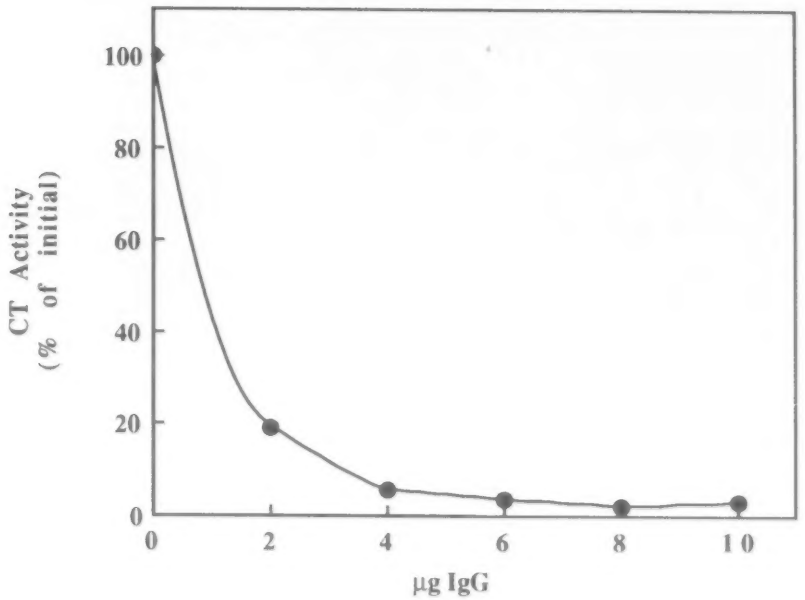

Fig. 9. Immunoprecipitation of CT from HeLa Cell Cytosol.

HeLa cells were digitonin-permeabilized for 5 min. Increasing amounts of affinity-purified α-CT IgG were added to the released cytosol. The complex formed between CT and its antibody was immunoprecipitated with Protein A-sepharose. CT activities were determined in the resulting supernatants after immunoprecipitation.

A similar immunoprecipitation of CT from rat hepatocyte cytosol using a non-specific IgG fraction showed no loss of CT activity (Jamil, *et al.*, 1992).

2.18.2. *Immunoprecipitation of CT from ^{32}P-Labeled Cells*

Confluent cells in 60 mm dishes were incubated in phosphorus-free medium for 30 min and then in phosphate-free medium containing 100 µCi/ml of ^{32}P-orthophosphate (200 µCi /dish) for 2 h. TPA (100 nM) was added to one dish and 0.1% DMSO to the control dish. After 1 h the medium was removed, cells were rinsed twice with cold PBS and permeabilized with digitonin. CT was immunoprecipitated from 450 µl of released cytosol and from RIPA buffer-solubilized cell ghosts as described by Jamil *et al.*, (1992) except that the preclearing step was ommitted and 0.5% bovine serum albumin was used to eliminate non-specific binding. The immunoprecipitated samples were prepared for SDS-PAGE by heating them at 80°C for 10 min with 75 µl Laemmli buffer. Proteins were separated by SDS-PAGE (10% polyacrylamide gel). The gel was dried and exposed to X-ray film for 20 h at -70°C in a film cassette with intensifier screens.

2.19. Downregulation and Immunoblotting of PKC

Cells were grown on 100 mm dishes and incubated in growth medium containing 1 µM TPA. At each time point cells from two dishes were rinsed with cold PBS, scraped into PBS and cells were pelleted for 15 seconds in a microfuge at 13,000 x g. The cell pellet was resuspended in 0.5 ml lysis buffer and sonicated with two 10 s bursts from a microtip probe. Unbroken cells and debris were pelleted by centrifugation at 13,000 x g in a microfuge for 15 s. The homogenate was centrifuged in a Beckman TL 100.2 rotor at 350,000 x g for 15 min in a Beckman TL 100 ultracentrifuge. The supernatant was saved as

cytosol. The pellet was resuspended in 0.5 ml lysis buffer containing 1% Triton-X 100, sonicated with 2 bursts of 10 s each and placed on ice for 15 min with occasional vortexing. The solubilized pellet was centrifuged for 15 min at 350,000 x g and the supernatant saved as the particulate fraction.

For SDS-PAGE 50 µg of protein from each of cytosol and particulate fraction, and 5 µl of pure bovine brain PKC (60 µg/ml) were prepared by adding equivalent amounts of Laemmli buffer and heating at 80°C for 10 min. The proteins in the samples were separated by SDS-PAGE (10% polyacrylamide gel), blotted onto a nitrocellulose membrane using a transfer buffer containing 25 mM Tris, 192 mM glycine, 20% methanol for 6 h at 0.2 A. The membrane was incubated in Blotto for 2 h, then in Blotto containing a monoclonal antibody to PKC (MC5 purchased from Amersham Corp.) at a 1:100 dilution overnight at 4°C with gentle shaking. The antibody solution was removed and the membrane rinsed four times with Blotto (10 min/rinse).

2.19.1. *Visualization of PKC Using ^{125}I-Protein A*

After the blotted membrane had been incubated with the primary antibody and rinsed, it was incubated in Blotto containing ^{125}I-Protein A (1.0 to 0.5 x 10^6 cpm/ml) for 1 h and rinsed 2 times with Blotto (5 min/rinse) followed by 2 rinses in TTBS. The membrane was air-dried and exposed to X-ray film at -70°C in a tightly-fitting film cassette with intensifier screens.

2.19.2. *Visualization of PKC Using Chemiluminescence*

The membrane was incubated in Blotto containing antibody to mouse immunoglobulin conjugated to horseradish peroxidase (1:500 dilution). After 1 h this was removed and the membrane was rinsed twice with Blotto (5 min /rinse), twice with TTBS (5 min/rinse) and finally twice with TBS (10

min/rinse). The membrane was placed in enhanced chemiluminescence (ECL) detection reagents (Amersham Corp.) for 2 min and sealed in a polyester pouch. This method of detection relies on the oxidation of luminol catalyzed by horseradish peroxidase, resulting in the emission of light (see Appendix B). The ECL was detected by exposing the sealed membrane to X-ray film (Kodak X-OMAT AR) for 15 s.

2.20. Immunocytochemistry of CT in HeLa Cells

2.20.1 *Immunofluorescence of CT in Intact HeLa Cells*

HeLa cells were plated sparcely (0.5 ml of 10^5 cells/ml in one chamber) in sterile Lab-Tek 4-chamber glass-slides (Nunc Inc., Illinois, USA) and used for immunofluorescence studies the next day. 4 chambers were plated to enable positive and negative controls to be carried out in parallel. The procedure was carried out as described in the following steps:

i) The cells were fixed for 20 min to the slides with 0.2 ml of Bouin's solution (2% paraformaldehyde, 5% picric acid, 5% sucrose in 0.2 M phosphate buffer, pH 7.6)

ii) The fixed cells were rinsed twice (5 min/rinse) with Hepes-buffered saline (HBS) containing 1 mM Ca^{2+}, 0.5 mM Mg^{2+} and 0.1 M glycine

iii) As CT is an intracellular antigen, the fixed cells were permeabilized for 3 min with HBS containing 1 mM Ca^{2+}, 0.5 mM Mg^{2+} and 0.1% Triton X-100, and subsequently rinsed once for one min with HBS-Ca^{2+}-Mg^{2+}.

iv) Non-specific sites were blocked with 0.2 ml of HBS-Ca^{2+}-Mg^{2+} containing 2% FBS for 20 min, followed by a rinse of one min with HBS-Ca^{2+}-Mg^{2+}.

v) In one chamber was placed 0.5 ml of HBS-Ca^{2+}-Mg^{2+} containing antibody to CT (1 : 100 dilution). As a control 0.5 ml of HBS-Ca^{2+}-Mg^{2+}

containing non-specific IgG (the eluant obtained after affinity-binding of immune serum on an affinity column for obtaining anti-CT IgG) at a dilution of 1 : 100 was placed in the second chamber. As a positive control 0.5 ml of HBS-Ca^{2+}-Mg^{2+} containing rabbit antibody to actin (1 : 100 dilution) was placed in the third chamber. In the fourth chamber was placed HBS-Ca^{2+}-Mg^{2+} without any antibody and this formed the negative control. The antibody solutions were allowed to sit in the slide chambers overnight at 4°C.

vi) The following day the antibody solutions were aspirated from all 4 chambers, and the cells were rinsed once with HBS-Ca^{2+}-Mg^{2+} for 5 min to dilute out unbound antibodies.

vii) The cells were incubated for 1 h at room temperature in the presence of HBS-Ca^{2+}-Mg^{2+} containing 2% goat serum and 2µl/ml of biotinylated goat anti-rabbit IgG as the secondary antibody.

viii) Subsequently, the solutions were aspirated and the cells rinsed once with HBS-Ca^{2+}-Mg^{2+} for one min, followed by a 30 min incubation with HBS containing 4 µg/ml Texas-red streptavaidin

ix) The Texas-red streptavidin solution was aspirated and the cells rinsed twice (5 min/rinse) with HBS-Ca^{2+}-Mg^{2+}.

x) The partitions between the chambers were removed, a coverslip placed on the slide and mounted with MOWIOL. Sealing was done with Revlon nail varnish.

xi) The cells were examined under a x40 oil immersion objective using a Zeiss microscope equipped with epifluorescent optics. Black and white photographs were taken using a high speed 35 mm, TMAX 400 Kodak film with a Contax 167 MT camera mounted on the microscope and equipped with an automatic photometer system.

2.20.2. *Immunofluorescence of CT in Digitonin-Permeabilized Cells*

For experiments dealing with detection of membrane-associated CT the cells were permeabilized on the glass slides with 0.5 ml of digitonin-buffer for 5 min after various treatments with agents. The cells were rinsed with PBS to remove all traces of cytosolic CT. Subsequently the cell-ghosts were fixed to the glass slides with Bouin's solution. All steps here on were as described in section 2.20.1.

2.21. Separation of DiC_8-PC by Thin Layer Chromatography

After lipid extraction the organic phase was spotted on silica gel plates which were developed in chloroform : methanol : acetic acid : formic acid : water (70 : 30 : 12 : 4 : 2). In this solvent system PC and dioctanoylphosphatidylcholine (DiC_8-PC) exhibited R_F values of 0.25 and 0.17, respectively.

DiC_8-PC was also separated according to a published procedure by Liscovitch *et al.* (1987) in which the organic phase was spotted on C_{18}-reversed phase TLC plates which were developed in methanol : acetonitrile : 0.25 M aqueous choline chloride (90.5 : 2.5 : 7). In this solvent system PC remained at the origin while DiC_8-PC exhibited an R_F value of 0.11.

2.22. Gas Liquid Chromatography of Fatty Esters.

A mixture of fatty acids were butylated for use as standards. 50 µl of a 10 mg/ml stock in dimethyl chloride were dried under nitrogen. The fatty acid mixture had the following composition; 8:0 (2%), 10:0 (3%), 12:0 (6%), 14:0 (12%), 16:0 (19%), 18:0 (25%) and 20:0 (33% by weight).

10 µl of a 0.5 M DiC_8 stock in DMSO was butylated to obtain the butyl ester of 8:0 as a single standard.

DiC_8-PC was separated from long chain PC as described in section 2.21 above. The area on the TLC plates corresponding to DiC_8-PC was scraped into a clean borosilicate screw-capped tube.

The above samples were trans-esterified by adding 1 ml of boron trifluoride (BF_3)-butanol[3] (10%, w/w) and immersing the tube in a boiling water bath for 15 min. The tube was cooled and 1 ml of water and 1 ml of hexane were added. The hexane phase was removed to a clean tube and extracted 3-5 times with a 10 times excess of water to remove the unreacted butanol. In the case of the DiC_8-PC sample, the extracted butylated 8:0 was evaporated at room temperature under nitrogen to a 20 µl volume (care was taken not to evaporate the sample to dryness as this could result in loss of some butyl 8:0).

For GLC separation of the butyl esters, 0.5 or 2 µl were injected into a Perkin-Elmer 8420 Capillary Gas Chromatograph. The oven temperature was programmed to be at 70°C for 15 min, after which it increased to 210°C at a linear rate of 2°C/min. The temperature remained at 210°C for a further 10 min. The total run time for each sample was 95 min. The injection and detection temperatures were 250°C. Helium at 30 pounds per sq. inch (psi) was

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[3]Initially, BF_3-methanol (14% BF_3 in methanol) was used to obtain methyl esters. However, the methyl ester of C_8 obtained was highly volatile and evaporated upon extraction and drying under nitrogen. Some methyl-C_8 was obtained when the hexane phase obtained after the trans-methylation reaction was evaporated to a volume of 20 µl under nitrogen by placing the tubes on ice, but this still resulted in considerable loss of methyl-C_8. The substitution of *n*-butanol for methanol in the borontrifluoride method prepares the butyl esters of the fatty acids. The losses associated with recoveries of short chain fatty acids such as 8:0 are avoided when butyl esters are used. Butyl esters are less volatile and less water soluble than are comparable short chain fatty acid methyl esters.

used as the neutral carrier gas. The 6 foot x 0.125 inch stainless steel column was packed with 10% diethyleneglycol succinate (DEGS) on a 100/120 mesh Supelcoport™. The butyl esters were detected by a flame ionization detector, and the resulting chromatogram printed on a Perkin-Elmer GP-100 Graphics Printer conected to the instrument. The chart speed was set at 2 mm/min and attenuation at 128 for the butyl fatty ester standards and at 32 for the other samples.

2.23. Separation of L and H Forms of CT by Glycerol Gradient Centrifugation

This procedure was performed essentially as described by Weinhold *et al.* (1989). Glycerol gradients (12 ml, linear 8 to 40%, v/v, in Buffer A) were prepared with a two-chamber gradient mixer. 0.5 ml cytosolic samples from various sources were layered over the gradients. Centrifugation was performed in a SW 40 rotor at 40,000 rpm for 14 h at 4°C in an L8-M ultracentrifuge. 0.5 ml aliquots were removed starting from the top and 60 µl assayed for CT for 30 min as described elsewhere in this chapter.

2.24. Transfection of HeLa Cells With the Yeast Phospholipid Methyltransferase (*PEM2*) Gene

2.24.1. Construction of the Expression Vector Containing the Yeast PEM2 Gene

A construct (pPM106G) with the yeast *PEM2* gene inserted into the plasmid vector pBR322 was obtained from Kodaki and Yamashita (1987). An expression vector containing *PEM2* was constructed in this laboratory by Dr. Zheng Cui according to the strategy outlined in Fig. 61. The EcoRI fragment containing the entire coding sequence of *PEM2* was spliced from pPM106G and

ligated into the EcoRI site of the retroviral expression vector pDOJ (Price *et al.*, 1987). The mouse murine leukemia virus long terminal repeat (Mo-MuLV LTR) provided the promoter for the *PEM2* gene. The SV40 early promoter and the Tn*5* *neo* gene, which transmits G418 resistance, were present downstream from *PEM2* and enabled selection of successfully transfected cell lines in the presence of G418.

2.24.2. *Transfection of Hela Cells*

HeLa cells were transfected with pDOJ/PEM2 and the pDOJ control vector by the calcium phosphate precipitation method (Wigler *et al.*, 1978). 8, 100 mm, dishes were plated with 1:15 diluted HeLa cells from confluent cultures the day before the transfection. The cells were fed again with growth medium 4 h before transfection. 10 µg of plasmid DNA which had been purified twice by caesium chloride gradient centrifugation by Dr. Zheng Cui were used for each dish. DNA was precipitated in 0.5 ml of 250 µM $CaCl_2$, 5 mM Tris-OH, 0.5 mM EDTA (pH 7.6) and added to an equal volume of 2x HEPES - buffered saline. The mixture was let to sit at room temperature for 30 min to allow the DNA to precipitate before being added dropwise to the cells. The dishes were swirled and put back in the incubator. The precipitates were left on the cells for 16 h and washed off with fresh growth medium containing 0.5 mg/ml G418 (gentamycin sulfate) and supplemented with 2 mM L-glutamine. Control cells were transfected with the pDOJ vector without any insert and grown in the same medium as pDOJ/PEM2 - transfected cells.

Cells transfected with pDOJ/PEM2 and pDOJ (controls) were selected in parallel. The cells were grown in growth medium containing 0.5 mg/ml G418 and 20 G418 - resistant clones were picked over the course of 7 to 30 days. The

cell lines were maintained in growth medium containing 0.5 mg/ml G418 and supplemented with 2 mM L-glutamine.

2.25. Harvesting of Cells

After the treatments specified in the figure legends, cells were rinsed twice with ice-cold PBS and scraped with a rubber policeman into 0.4 ml x 2 of PBS. Subsequently, the cells were sonicated with a microtip probe for 10 sec x 2, and 0.5 ml of the homogenate taken for lipid extractions. Aliquots from the remaining homogenate were taken for protein estimations.

2.26. Data Analyses

CT assays were performed in duplicate and expressed as an average of the two values, as were protein and phospholipid measurements. LDH assays were performed in triplicate and expressed as an average of the three values.

Linear regression analyses (performed on a Mackintosh Plus computer using the Cricket graph program) were used to determine the best fit of lines through data points on standard curve plots.

Where appropriate significance of difference of data between the means of two sets of data was determined by the Student's *t*-test.

Normalization of data - Since there was some variation in absolute values obtained from apparently identical experiments, the data from some experiments were normalized. The experimental values obtained were multiplied by a numerical factor which was obtained by dividing the *average* control value by the *individual* control value.

CHAPTER 3 RESULTS

3.1 STIMULATION OF PC BIOSYNTHESIS BY TPA

In 1979 Kinzel *et al.* showed that TPA was the most potent phorbol ester in stimulating the incorporation of [*methyl*-^{3}H]choline into the choline-containing lipids of HeLa cells. The tumor-promoting activities of TPA correlated positively with its ability to stimulate PC biosynthesis. This stimulation of PC biosynthesis was, therefore, used as a diagnostic tool to assess the potency of other phorbol esters. 100 nM TPA caused a maximal effect within 2 h. 1 and 10 nM TPA concentrations exhibited significant effects at later times. Therefore, TPA has been used at a 100 nM concentration throughout this study. This concentration has also been used consistently in previous work from this laboratory.

3.1.1 *Incorporation of [methyl-^{3}H]choline into PC*

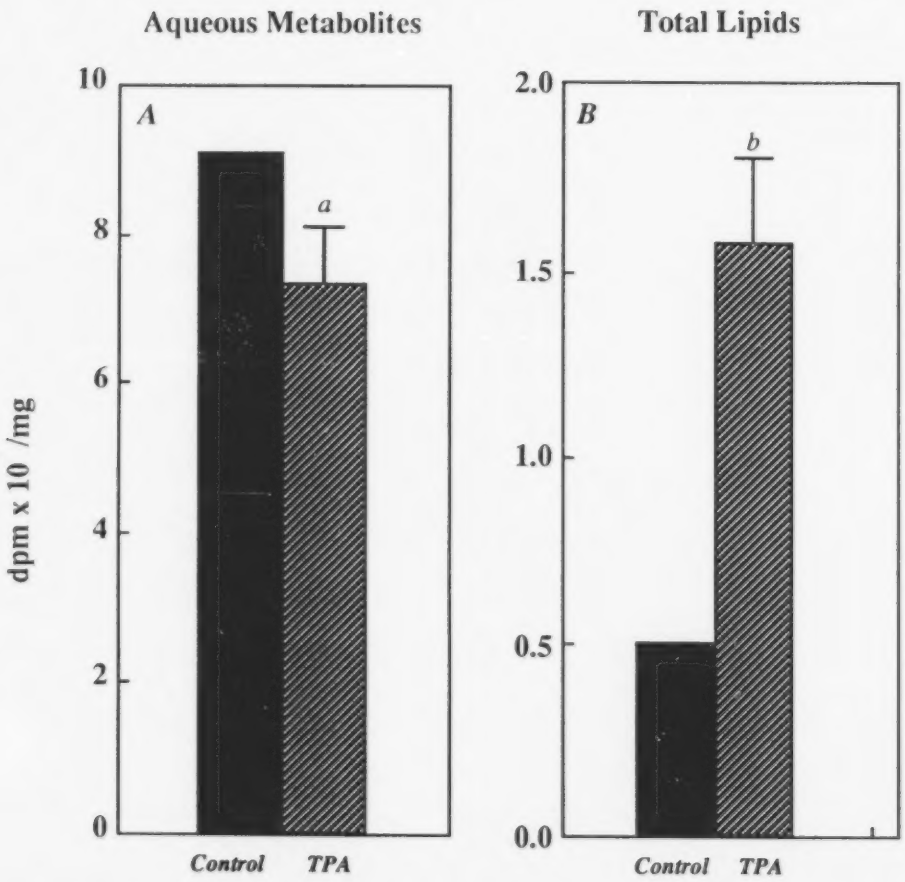
Pulse-chase experiments showed stimulation of PC biosynthesis by TPA. Confluent HeLa cells were prelabeled with [*methyl*-^{3}H]choline for 1 h. The labeled medium was removed and the cells incubated for a further 1 h in medium containing 0.1% DMSO *(Control)* or TPA *(100 nM)*. Total cellular lipids were extracted and the radiolabel in the aqueous metabolites and the total lipid fraction determined. There was a three-fold stimulation of incorporation of tritiated choline into the total lipid fraction (radiolabeled PC constituted greater than 90% of the radiolabel in the total lipid fraction) with a concomitant decrease of radiolabeled aqueous metabolites (Fig. 10). These results are in agreement with those of Pelech, Paddon and Vance (1984). The

Fig. 10. Effect of TPA on Choline-Containing Metabolites.

Confluent HeLa cells were prelabeled for 1 h in medium containing 2 μCi /ml [^{3}H]choline chloride. The labeled medium was removed, cells were rinsed thrice and incubated for a further 1 h with medium containing 0.1% DMSO (*Control*) or TPA (*100 nM*). The chase medium was removed, and the cells were rinsed twice with ice-cold PBS. Lipids were extracted from the cells. Radiolabel was determined in choline-containing metabolites in the aqueous phase (***A***) and total lipid fraction (***B***). These data (mean ± S.D.) represent duplicate determinations from four experiments, after normalization to control values and are expressed on a per mg protein basis.

a $0.0005 \leq p \leq 0.005$ (n=4)

b $p \leq 0.0005$ (n=4)


Aqueous Metabolites
Total Lipids
A
B
a
b
10
8
6
4
2
0
2.0
1.5
1.0
0.5
0.0
dpm x 10 -5 /mg
Control
TPA
Control
TPA

decrease in radiolabeled aqueous metabolites in TPA-treated HeLa cells as compared to untreated cells has previously been shown by Pelech *et al.*, (1984) to be from phosphocholine (the major labeled aqueous metabolite) with no significant difference in labeled choline. Radiolabeled CDP-choline constituted less than 5% of the radiolabel in the aqueous phase.

3.1.2. Time-Course of TPA-Stimulated PC Biosynthesis

The stimulation of PC biosynthesis by TPA over a 2 h time period was investigated. HeLa cells were prelabeled for 1 h with 2 μCi/ml [*methyl*-^{3}H]choline chloride and subsequently chased in the presence of 0.1% DMSO (Control) or TPA (100 nM). At various times cells were harvested, lipids extracted and the radioactivity in the total lipid fraction determined. There was a 30 min lag before an increased incorporation of [^{3}H]choline was seen in TPA-treated cells (Fig. 11).

3.1.3 *Translocation of CT*

Since TPA has been shown by Paddon and Vance (1980) not to affect choline transport into HeLa cells, or choline kinase or cholinephosphotransferase activities, it appeared that the TPA-mediated stimulation of PC biosynthesis was via CT. Subsequently, Pelech, Paddon and Vance (1984) showed that CT was activated in TPA-treated HeLa cells by causing a redistribution of the enzyme activity from an inactive cytosolic form to an active membrane-associated form. This finding is confirmed in Fig. 12.

a) <u>Subcellular distribution of CT after Dounce homogenization of HeLa cells</u>: subcellular distribution of CT was studied by breaking cells open by Dounce homogenization after various treatments (Fig. 12). This method of rupturing cells, however, gave inconsistent results with respect to the

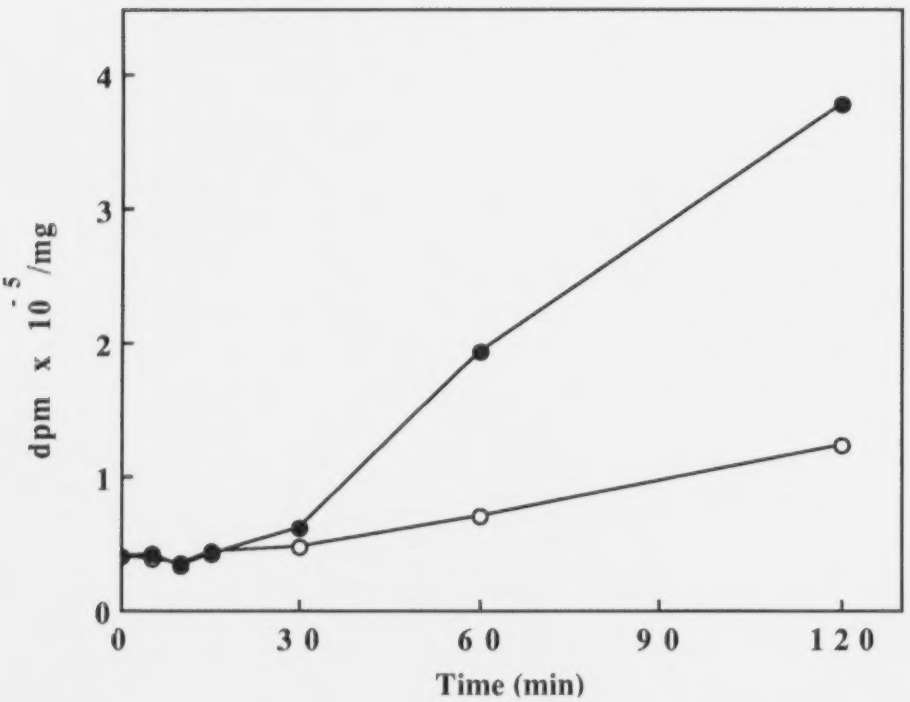


Fig. 11. Time-Course of TPA-Stimulated PC Biosynthesis. Confluent HeLa cells were prelabeled for 1 h with 2 μCi/ml [^{3}H]choline chloride (4 μCi/dish), rinsed thrice with warm medium and incubated in chase medium containing 0.1% DMSO (control) or TPA (100 nM). Cells were harvested after various times, lipids extracted and radiolabel in total lipids determined. The data are expressed on a per mg protein basis, are means of duplicate values and representative of three experiments.

absolute values obtained as well as the total (cytosolic plus microsomal) activities obtained. The results in Fig. 12 are, therefore, expressed as % of total cytosolic and microsomal activities. Moreover, the extent of TPA-elicited increase in microsomal CT activity (less than two-fold over that in controls) after 1 h did not correlate with the three-fold stimulation of PC biosynthesis observed in Fig. 10 at the same time.

Unsaturated fatty acids such as oleate have been reported by Pelech, Paddon and Vance (1984) and Cornell and Vance (1987a) to cause dramatically the translocation of CT from the cytosol to membranes in HeLa cells. Fig. 13 shows the translocation of CT activity from the cytosol to microsomes upon incubation of HeLa cells with 0.35 mM oleate, and the reversal of this phenomenon by 10 mg/ml albumin. Since oleate causes such a dramatic translocation of CT activity, this treatment was used frequently as a positive control in studies dealing with CT translocation.

b) the nitrogen cavitation method: A different method of rupturing cells was tried. Cells were put in a mini-bomb cell disrupter chamber and subjected to high pressure (500 psi of nitrogen for 5 min) as described in Section 2.13.2. The resulting homogenate was sub-fractionated into cytosolic and microsomal fractions, and the CT activities determined. Fig. 14 shows the results of CT activities obtained in cytosolic and microsomal fractions after 1 h TPA-treatment of HeLa cells. Table 1 summarizes the results of cytosolic and microsomal activities expressed as total activities (nmol/min). The absolute CT activity values obtained when cells were ruptured by nitrogen cavitation were consistent between samples (as shown by the values from controls in Table 1) and also between experiments. However, the expected TPA-elicited translocation was not apparent, as both cytosolic and microsomal activities

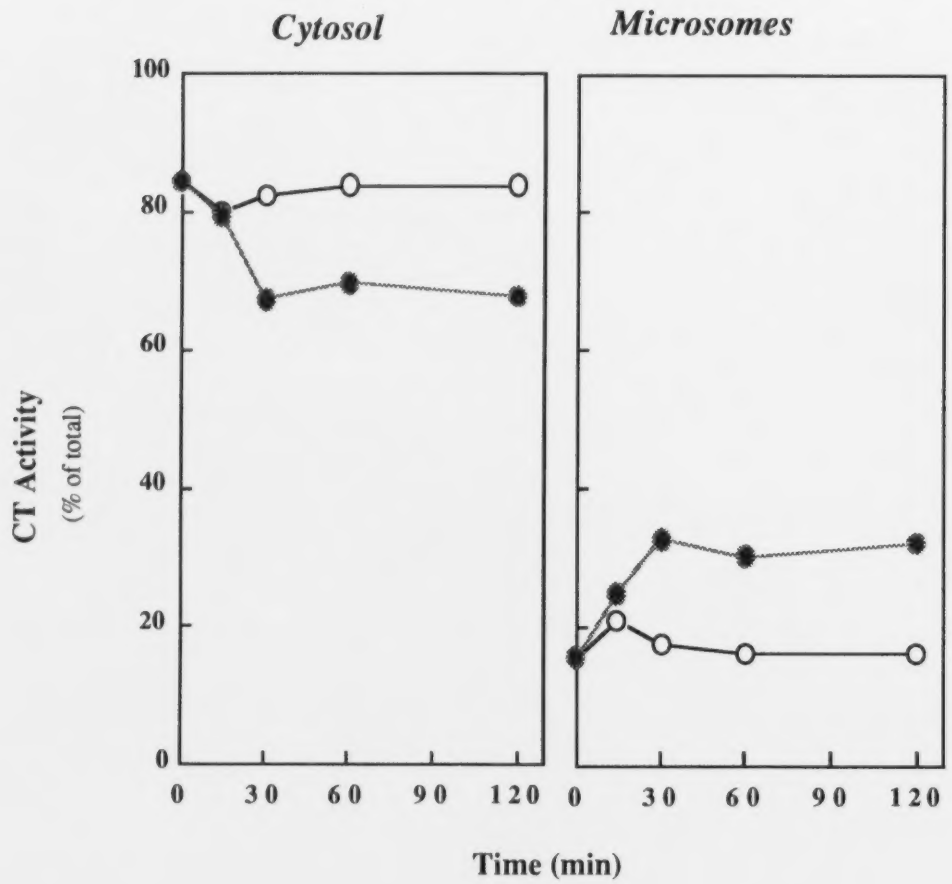


Fig. 12. TPA-Elicited Translocation of CT Activity From the Cytosol to Microsomes. HeLa cells were incubated ± 100 nM TPA. At various times cells were harvested, homogenized and fractionated into cytosol and microsomes. CT activities were determined and are expressed as % of total cytosolic + microsomal activities. Control values of CT activities in the cytosol and microsomes were 8.61 ± 1.40 and 4.10 ± 0.52 nmol/min/mg protein, respectively (n=6). Open and solid symbols represent samples from untreated and TPA-treated cells, respectively.

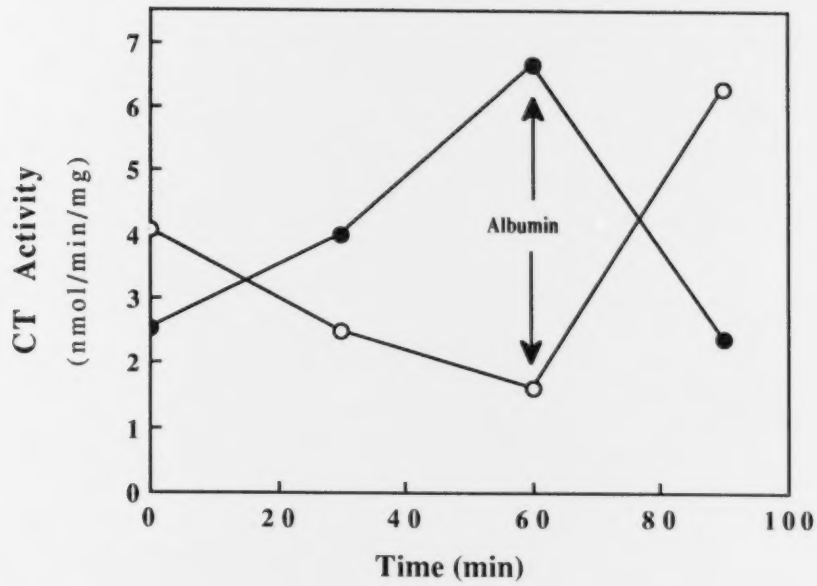


Fig. 13. Translocation of CT Activity to Microsomes in the Presence of Oleate and the Reversal to Cytosol by Albumin. HeLa cells in growth medium were treated with 0.35 mM oleate for various times. At 60 min albumin was added to a final concentration of 10 mg/ml. The cells were harvested at the times shown, homogenized and fractionated into cytosol and microsomes. Specific CT activities were determined in the cytosolic (open symbols) and microsomal (solid symbols) fractions. The results are representative of three different experiments.

were increased upon TPA-treatment. When the activities were expressed as % of total, no TPA-elicited effect was apparent (Table 1).

c) the digitonin-permeabilization method: Since both of the methods for rupturing cells described above are harsh, a relatively mild procedure (detergent-permeabilization) was employed. Detergent-permeabilization is the least destructive method of rupturing cells (Mackall *et al.*, 1979). A buffered digitonin solution was used to permeabilize cells that had been treated with 100 nM TPA for 1 h. LDH activity was determined as a marker of cytosolic release (Fig. 15). Fig. 16 shows the total protein and CT activities that were determined in the released cytosolic fraction and retained particulate fraction. Fig. 17 shows the results of an experiment in which HeLa cells were incubated in the absence (***A***) or presence (***B***) of 100 nM TPA for 1 h, and then permeabilized with digitonin for 5 min. The released cytosolic and retained CT activities were determined. There was a small but significant decrease in cytosolic CT from TPA-treated cells as compared to controls. Moreover, the corresponding increase in particulate CT activity from TPA-treated cells was three-fold that in control cells. This correlated well with the three-fold stimulation of PC biosynthesis seen in Fig. 10.

The digitonin-permeabilization technique was also used to investigate the translocation of CT elicited by the unsaturated fatty acid, oleate (Fig. 18). Incubation of HeLa cells in the presence of 0.35 mM oleate for 1 h caused a decrease in the digitonin-mediated release of cytosolic CT from 7.56 in controls to 2.84 nmol/min/mg in oleated-treated cells. There was a corresponding increase in particulate CT activity from 0.351 in controls to 4.2 nmol/min/mg in oleate-treated cells. This translocation of CT was reversed to 6.98 and 0.354 nmol/min/mg in the cytosolic and particulate fractions, respectively, within 30 min upon the addition of 10 mg/ml albumin to the incubation medium.

Fig. 14. Effect of Nitrogen Cavitation on the Subcellular Distribution of CT Activity in HeLa Cells. HeLa cells were incubated in the absence (open symbols) or presence (solid symbols) of 100 nM TPA for 1 h. The cells were harvested, subjected to nitrogen cavitation (500 psi for 5 min) and fractionated into cytosol and microsomes. CT activities were determined in the cytosol and microsomes and are expressed as specific activity.

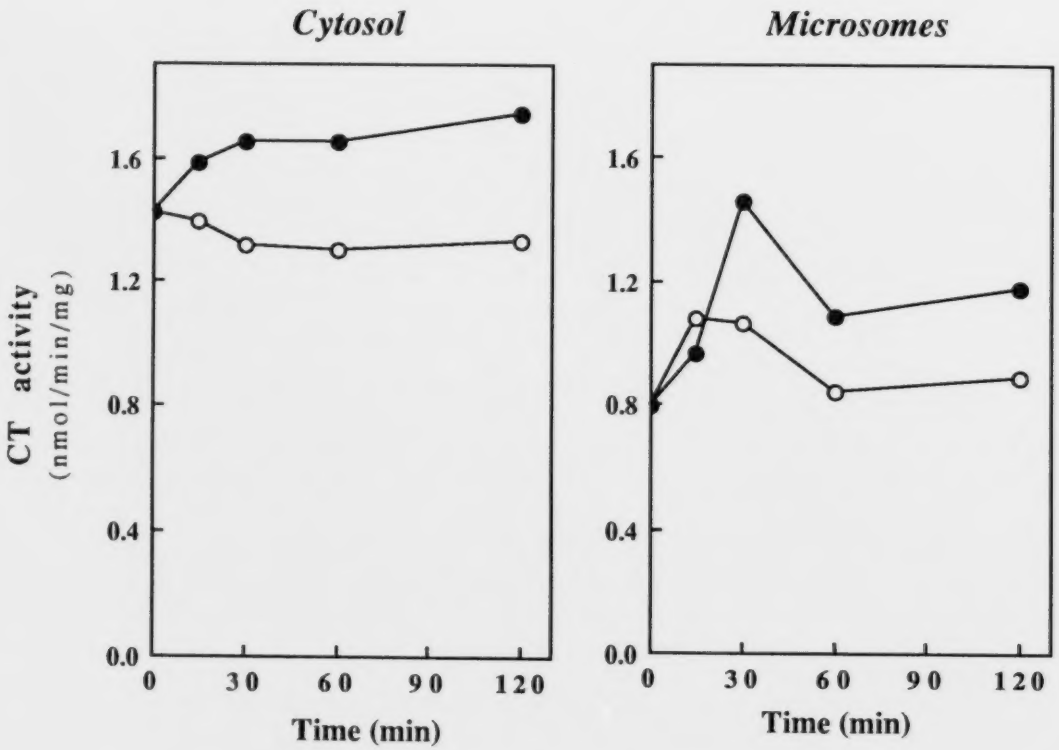

Cytosol
Microsomes
CT activity
(nmol/min/mg)
0.0
0.4
0.8
1.2
1.6
0
30
60
90
120
Time (min)

Table 1. Subcellular Distribution of CT Activity in HeLa Cells Treated With TPA and Ruptured by Nitrogen Cavitation. HeLa cells were incubated in the absence (Control) or presence of 100 nM TPA. At various times cells were harvested, subjected to nitrogen cavitation (500 psi for 5 min) and fractionated into cytosol and microsomes. CT activities were determined. The results are representative of two identical experiments.

Time (min)	Total (cytosolic + microsomal) Activity (nmol/min)		CYTOSOLIC CT ACTIVITY				MICROSOMAL CT ACTIVITY			
			Control		TPA		Control		TPA	
	Control	TPA	nmol/min/mg	% of total activity	nmol/min/mg	% of total activity	nmol/min/mg	% of total activity	nmol/min/mg	% of total activity
0	0.11		1.43	73	1.43	73	0.79	27	0.79	27
15	0.12	0.13	1.39	66	1.58	67	1.07	34	0.96	33
30	0.12	0.15	1.32	65	1.65	58	1.06	35	1.46	42
60	0.11	0.14	1.30	70	1.65	66	0.84	30	1.08	34
120	0.11	0.16	1.33	65	1.74	63	0.89	35	1.18	37

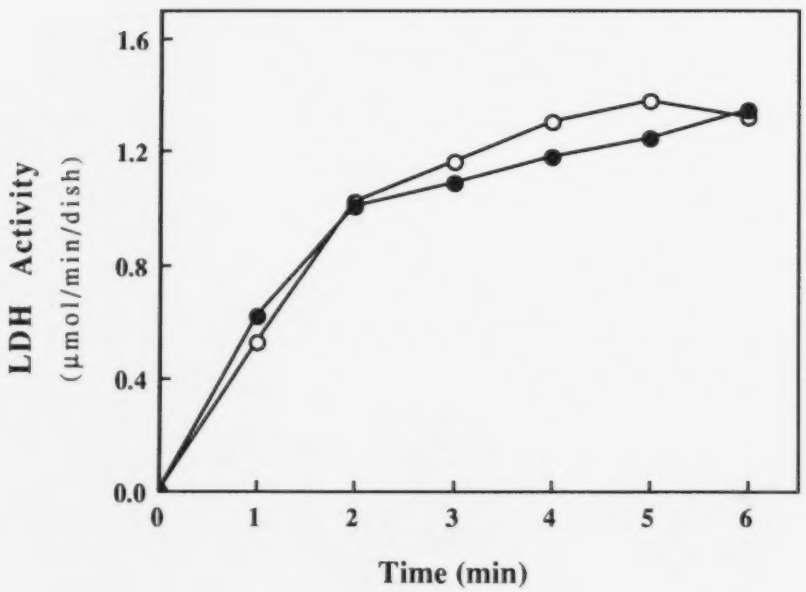


Fig. 15. Digitonin-Mediated Release of LDH Activity in HeLa Cells. HeLa cells were incubated in the absence (open symbols) or presence (solid symbols) of 100 nM TPA for 1 h and permeabilized with digitonin for various times. The released cytosolic LDH activities were determined. The results are averages of duplicate determinations and are representative of three separate experiments.

Fig. 16. Digitonin-Mediated Release of CT From TPA-Treated Cells. Confluent HeLa cells were treated with medium containing 0.1% DMSO (open symbols) or 100 nM TPA (solid symbols) for 1 h. The medium was removed, cells were rinsed twice with PBS and permeabilized with digitonin for the times indicated. The released cytosolic (*A*) and retained particulate (*B*) fractions were assayed for protein and total CT activity. Insets show profiles of total mg protein released and retained respectively, per dish. The data are means of duplicate values from one of three similar experiments. CT activity is expressed as total nmol CDP-choline formed/min/dish of cells.

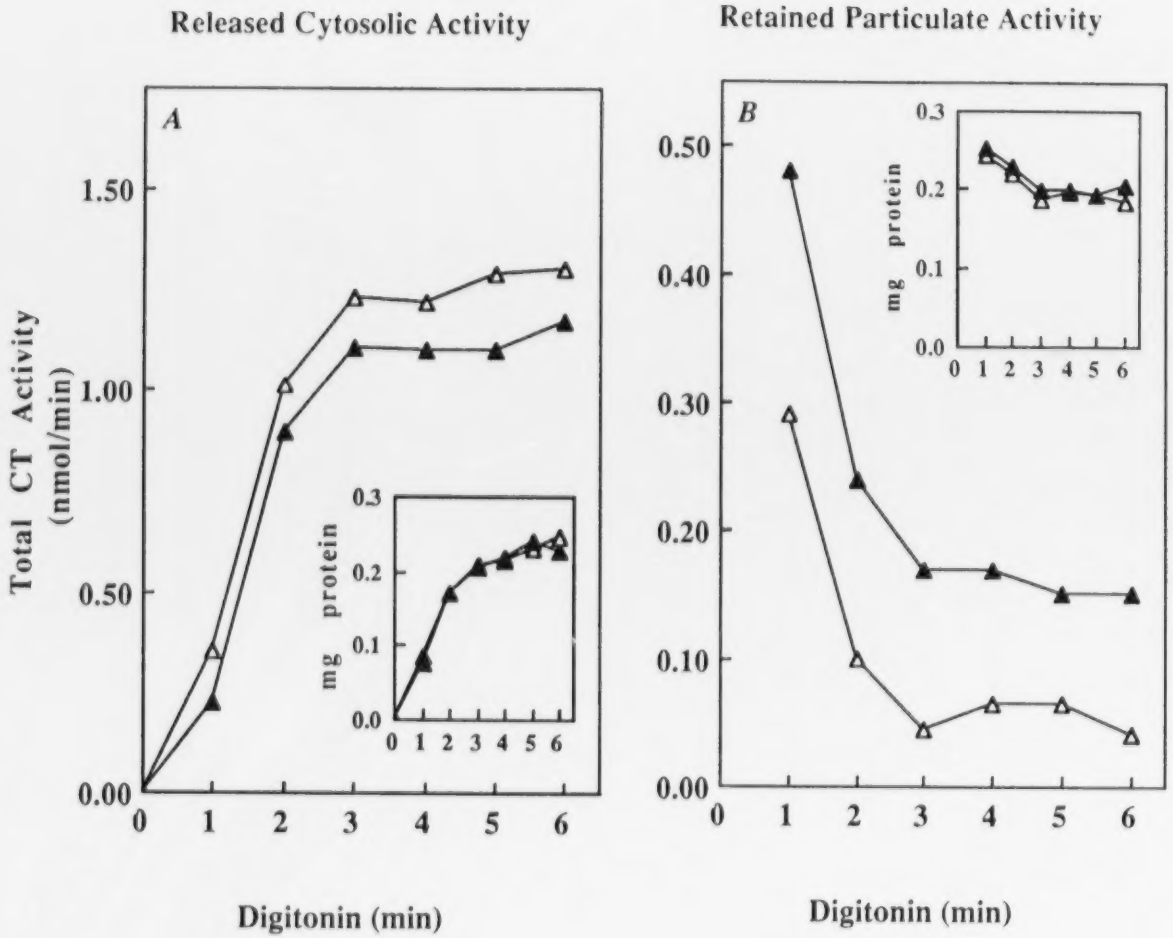
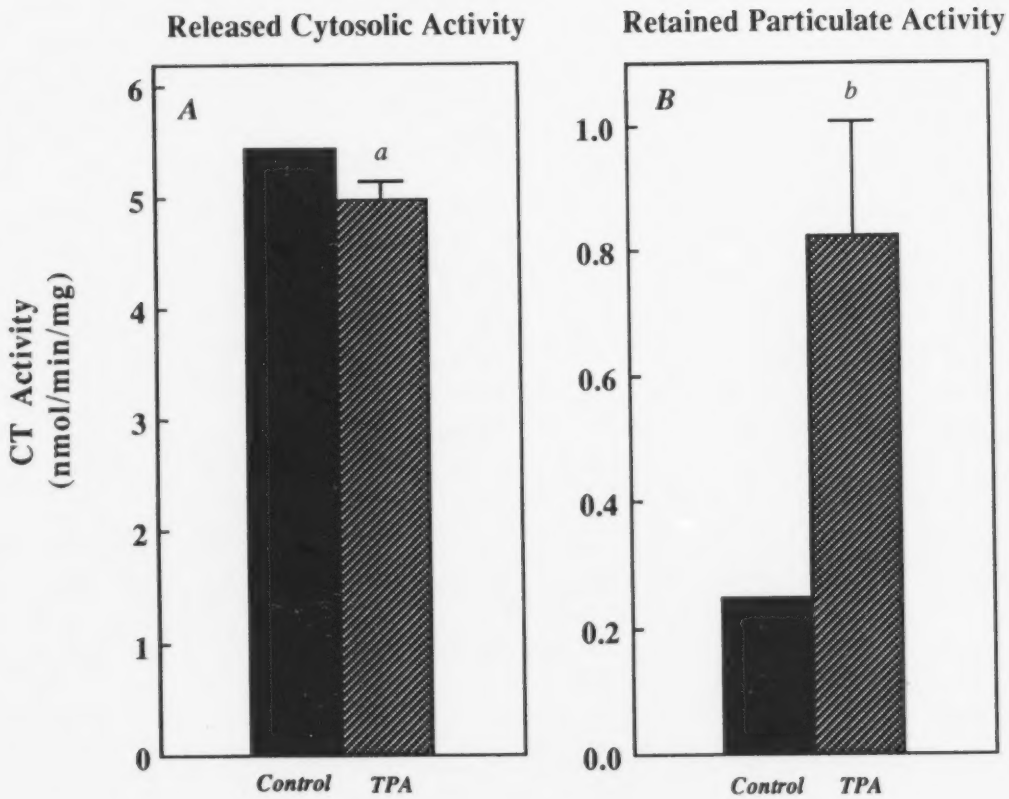

Released Cytosolic Activity
Retained Particulate Activity
A
B
Total CT Activity (nmol/min)
mg protein
Digitonin (min)
Digitonin (min)

Fig. 17. TPA Causes a Three-Fold Increase in Membrane-Associated CT Activity. HeLa cells were treated with 0.1% DMSO (*control*) or *TPA* (100 nM) for 1 h, rinsed twice with cold PBS and then permeabilized with digitonin for 5 min. Digitonin-released cytosol (***A***) and retained particulate (***B***) CT activities were assayed and expressed on a per mg protein basis. These data (mean ± S.D.) represent duplicate determinations from four experiments and are plotted after normalization to control values.

a $p \leq 0.005$ (n=4)

b $p \leq 0.0005$ (n=4)


Released Cytosolic Activity
Retained Particulate Activity
CT Activity
(nmol/min/mg)
A
B
a
b
6
5
4
3
2
1
0
1.0
0.8
0.6
0.4
0.2
0.0
Control
TPA
Control
TPA

The utility of the digitonin-permeabilization method for studying CT translocation was further demonstrated using a different cell line. The REF 52 cell line is a Fisher rat embryo-derived cell line. It is immortalized, nontransformed and nontumorigenic. Agonist (e.g. vasopressin)- or TPA-stimulation of this cell line causes a 3 to 4-fold stimulation of PC hydrolysis (Cabot *et al.*, 1988). The cells were treated with 100 nM TPA for 30 min and permeabilized with digitonin for various times. Fig. 19 shows the digitonin-released LDH activities. CT activities were determined in the released cytosolic contents and the retained particulate fractions as shown in Fig. 20. Similar studies using Dounce homogenization to rupture cells and obtain sub-cellular fractions were largely unsuccessful as they gave inconclusive and inconsistent results.

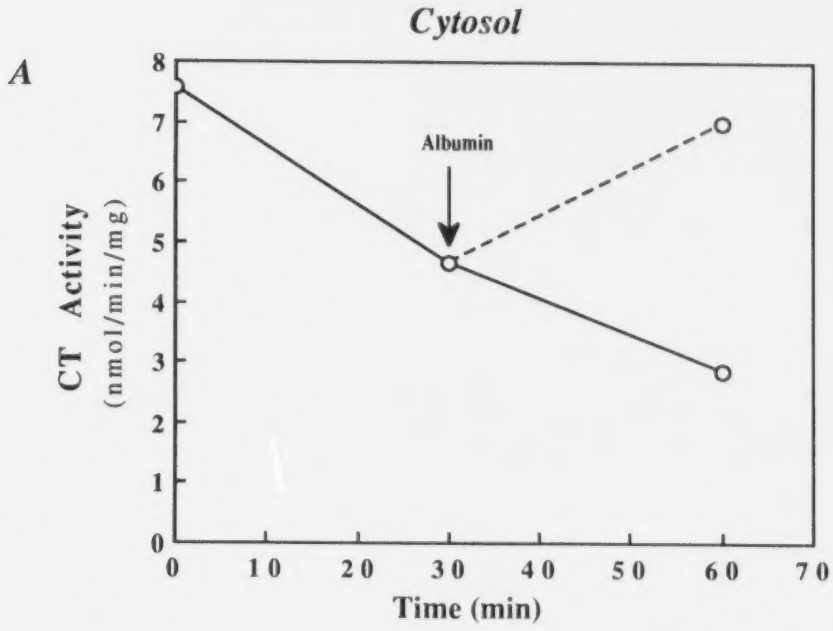
3.1.4. Time-Course of TPA-Elicited CT Translocation

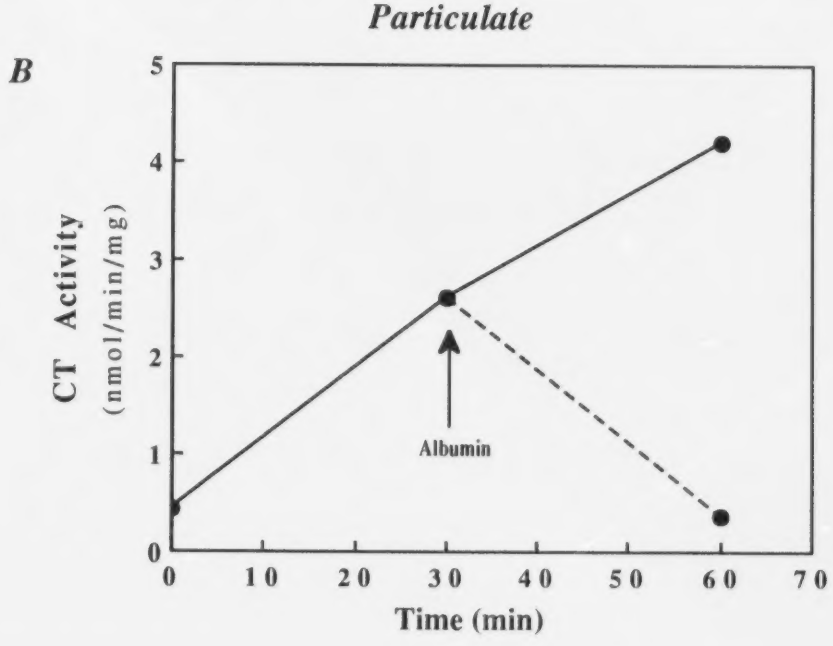
The time-course of CT translocation to membranes in response to TPA-treatment was investigated (Fig. 21). HeLa cells were incubated in the presence of 0.1% DMSO (Control) or TPA (100 nM). At various times the cells were digitonin-permeabilized and CT activities determined in the particulate fractions. There was a lag of approximately 10 min before an increase in membrane-associated CT was observed in TPA-treated cells. The increase in CT translocation to membranes was maximal at 30 min and remained elevated over the 2 h time period of TPA-treatment.

3.1.5 *Immunoblotting of CT*

Translocation of CT to membranes was visualized by immunoblotting. Earlier attempts to detect membrane CT were largely unsuccessful as seen in Fig. 22 although cytosolic CT was detectable. Since the amount of CT activity

Fig. 18. Oleate-Elicited CT Translocation to Membranes and its Reversal by Albumin. HeLa cells were treated with 0.35 mM oleate. At 30 min albumin at 10 mg/ml (dashed lines) was added to the incubation medium. The cells were permeabilized with digitonin for 5 min and specific CT activities determined in the released cytosolic (*A*) and retained particulate (*B*) fractions. The dashed lines show the reversal of oleate-induced CT translocation by albumin, whereas the solid lines represent cells that were incubated in the presence of oleate alone.


A
Cytosol
CT Activity
(nmol/min/mg)
Albumin
0
1
2
3
4
5
6
7
8
0 10 20 30 40 50 60 70
Time (min)


B
Particulate
CT Activity
(nmol/min/mg)
Albumin
0
1
2
3
4
5
0 10 20 30 40 50 60 70
Time (min)

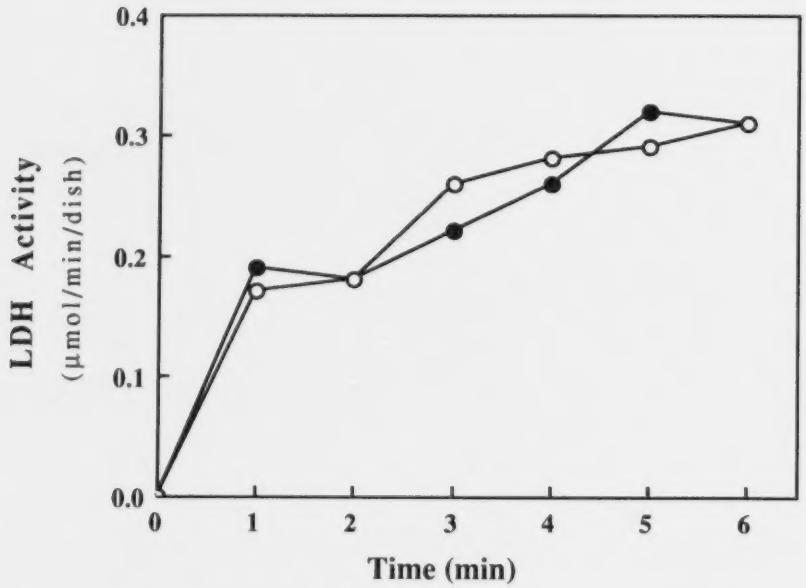
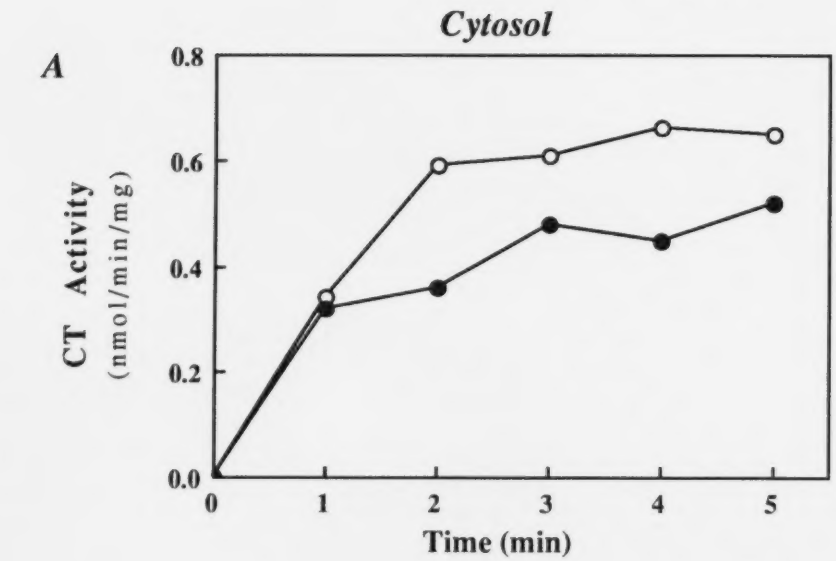
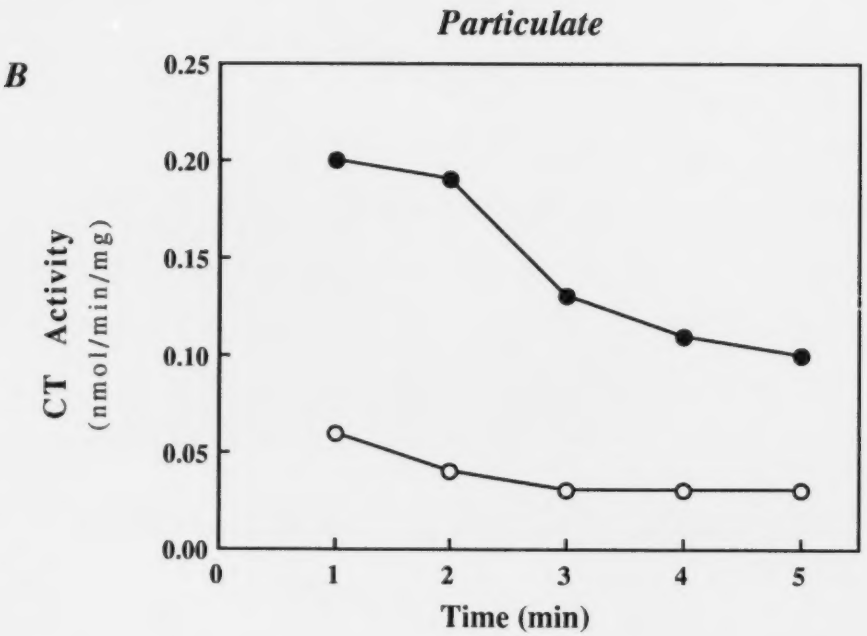


Fig. 19. Release of LDH Activity From REF 52 Cells by Digitonin.

REF 52 cells were incubated in the absence (open symbols) or presence (solid symbols) of 100 nM TPA for 30 min. The cells were permeabilized with digitonin for various times. The released LDH activities were determined in triplicate.

Fig. 20. Subcellular Distribution of CT in TPA-Treated REF 52 Cells. REF 52 cells were incubated in the absence (open symbols) or presence (solid symbols) of 100 nM TPA for 30 min. The cells were permeabilized with digitonin for various times. Specific CT activities were determined in the released cytosolic (*A*) and the retained particulate (*B*) fraction.


A
Cytosol
CT Activity
(nmol/min/mg)
0.8
0.6
0.4
0.2
0.0
0
1
2
3
4
5
Time (min)


B
Particulate
CT Activity
(nmol/min/mg)
0.25
0.20
0.15
0.10
0.05
0.00
0
1
2
3
4
5
Time (min)

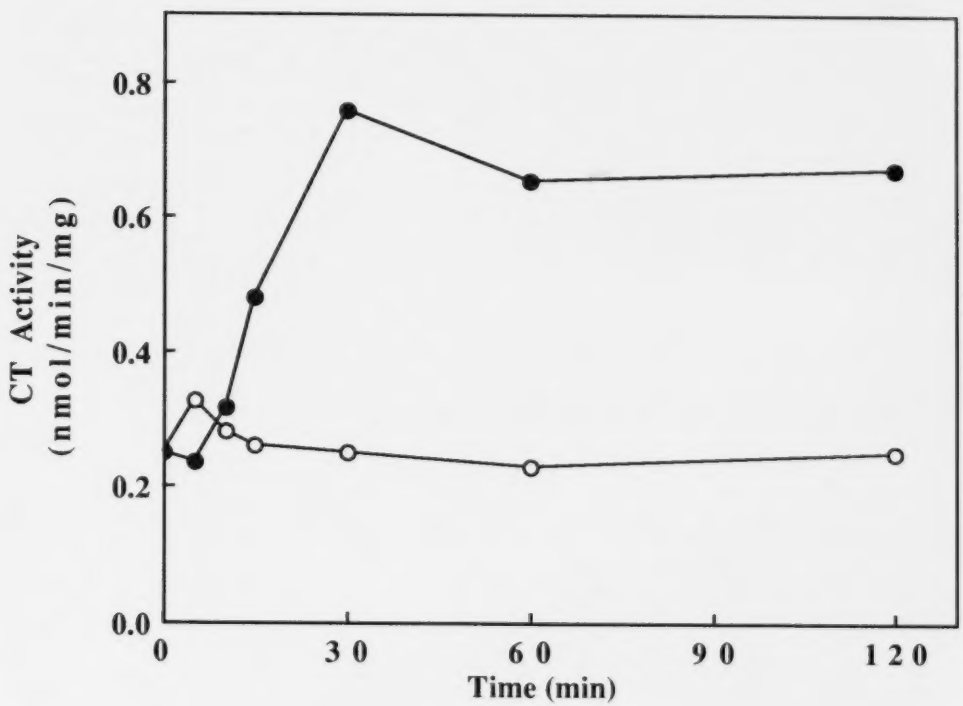


Fig. 21. Time-course of TPA-elicited CT translocation.

Confluent HeLa cells were incubated in medium containing 0.1% DMSO (open symbols) or 100 nM TPA (solid symbols). At various times incubation medium was removed, cells permeabilized with digitonin for 5 min and particulate CT activity determined. The data are expressed on a per mg protein basis, are means of duplicate values and representative of three experiments.

associated with membranes is only 3% of the total cellular activity (as seen by digitonin-permeabilization experiments), more sensitive means of detection were employed. This was made possible by the commercial availability of a detection system using chemiluminescence. The principle of the method is described in Appendix B. Fig. 23 shows that upon TPA-treatment of HeLa cells, the amount of membrane-associated CT increased with respect to that in untreated cells. Thus the translocation of CT activity is confirmed by the translocation of the enzyme protein itself.

Several unsuccessful attempts were made to demonstrate the translocation of CT by immunoprecipitating CT from [^{35}S]methionine-labeled cells. Upon fluorography of the gels numerous labeled bands were seen, many overlapping with CT, making it impossible to identify CT.

Immunoblotting was also used to demonstrate oleate-elicited translocation of CT to membranes and its reversal by albumin (Fig. 24).

3.2 DIACYLGLYCEROL AS A TRANSLOCATION SIGNAL FOR CT

The mechanism of translocation of CT to membranes in TPA-treated cells was investigated. TPA is known to increase the formation of DG in various cell types (Daniel *et al.*, 1986; Besterman *et al.*, 1986; Glatz *et al.*, 1987; Cabot *et al.*, 1988). In addition, enhanced binding of CT to DG-enriched membranes has been demonstrated by Cornell and Vance (1987). The role of DG in the translocation of CT in TPA-treated cells was, therefore, investigated.

3.2.1 *Accumulation of labeled DG in TPA-treated cells*

As a first step towards evaluating the role of DG as a possible translocation signal for CT, experiments were carried out to demonstrate an

Fig. 22. Immunoblotting of CT. HeLa cells were incubated in the absence (lanes 1 and 3) or presence (lanes 2 and 4) of 100 nM TPA for 1 h, and digitonin-permeabilized to obtain the cytosolic and particulate fractons. 400 μg of cytosolic (lanes 1 and 2) and 800 μg of solubilized particulate (lanes 3 and 4) were subjected to SDS-PAGE (10% separating gel) and blotted onto an Imobilon-P membrane. The membrane was subjected to immunoblot analysis using a rabbit polyclonal antibody to CT. ^{125}I-protein A was used to visualize CT. X-ray film was exposed to the dried gel for 7 days at -70 °C with intensifying screens. The first lane had purified CT.

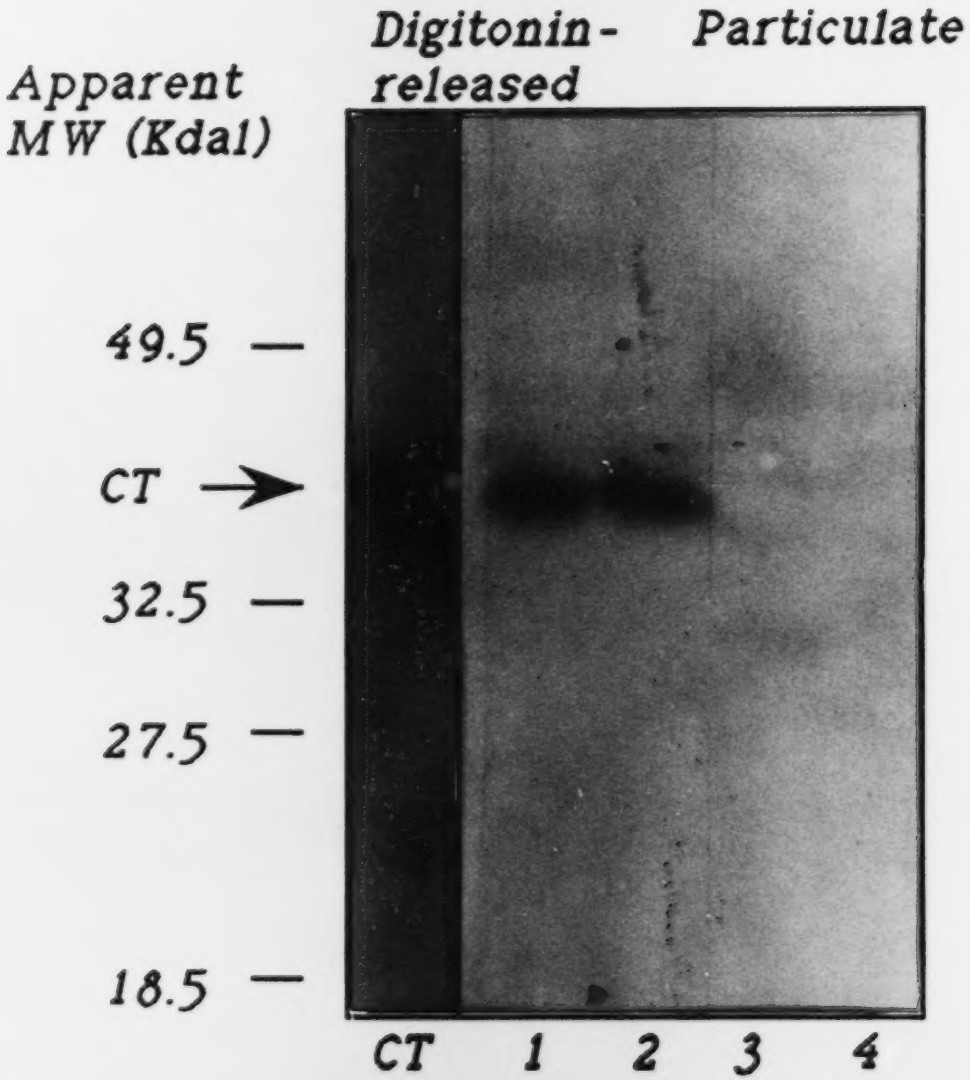

Digitonin-released
Particulate
Apparent MW (Kdal)
49.5
CT
32.5
27.5
18.5
CT
1
2
3
4

Fig. 23. Immunoblot Showing TPA-Elicited CT Translocation. Digitonin-released cytosolic (panel **A**) and particulate (panel **B**) samples from untreated (*1*) or TPA-treated (*2*) cells were fractionated by SDS-PAGE (10% polyacrylamide gel), blotted onto an Immobilon-P membrane and subjected to immunoblot analysis using a rabbit polyclonal antibody to CT and a chemiluminescent method for visualizing CT. 100 µg cytosolic fractions and 400 µg particulate fractions were loaded onto the gel. The first lane in panel **A** shows pure CT.

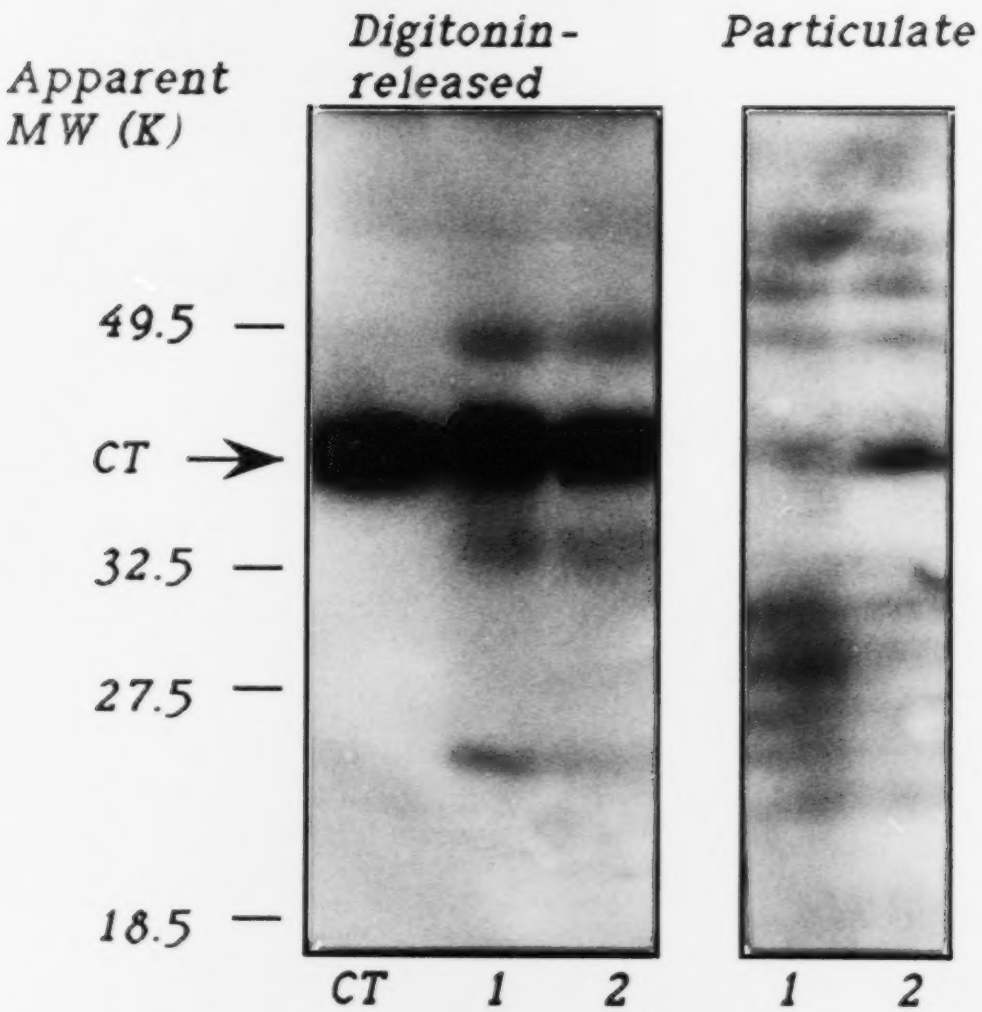
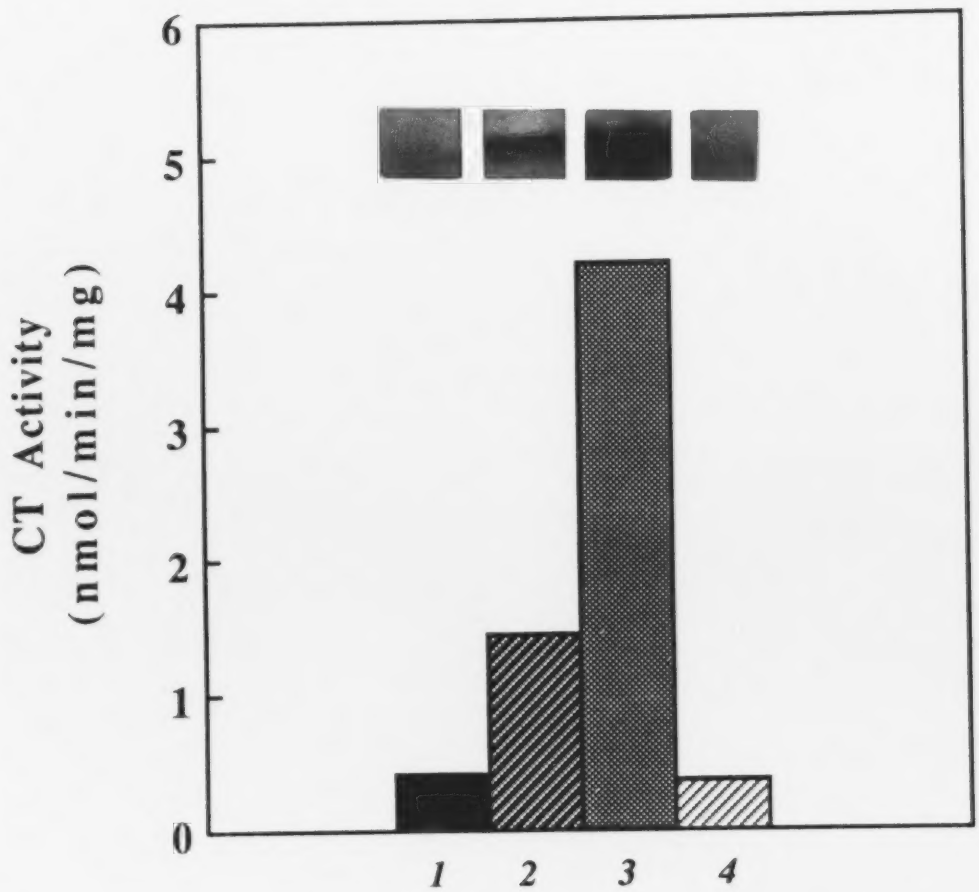

Digitonin-released
Particulate
Apparent MW (K)
49.5
CT
32.5
27.5
18.5
CT
1
2
1
2

Fig. 24. Immunoblot Analysis of CT Translocation Caused by TPA and Oleate. HeLa cells were incubated in the presence of 100 nM TPA for 1 h (lane 2), 0.35 mM oleate for 1 h (lane 3), and 0.35 mM oleate for 30 min. followed by 10 mg/ml albumin for a further 30 min. (lane 4). Lane 1 shows a sample from untreated control cells. The cells were digitonin-permeabilized for 5 min to obtain the particulate fraction. 400 µg of solubilized particulate fractions were subjected to SDS-PAGE (10% separating gel) and blotted onto an Imobilon-P membrane. The membrane was subjected to immunoblot analysis using a rabbit polyclonal antibody to CT and CT was visualized using the ECL method. The results of the immunblot are shown as insets corresponding to the CT activities represented in the bar graph.


CT Activity
(nmol/min/mg)
6
5
4
3
2
1
0
1
2
3
4

increase in DG upon treating cells with TPA. Initially, this was investigated by monitoring the increase of DG by labeling cells with a suitable radiolabelled precursor. For this purpose [2-^{3}H]glycerol was chosen since this precursor would enable most lipids to be labeled and the effect of TPA on DG labeling could be monitered. In initial experiments cells were prelabeled for 4 h, and chased ± 100 nM TPA for various times in the absence of labeled glycerol. Subsequently, the cells were harvested, the lipids separated by TLC and subjected to liquid scintillation counting. There was no difference in the label in DG from TPA-treated cells as compared to the labeled DG from untreated controls. A report by Glatz *et al.* (1987) showed that the DG produced in HeLa cells due to TPA-treatment was derived from PC (likely via a phospholipase C hydrolysis of PC). In the light of this observation it appeared that the PC in the [2-^{3}H]glycerol-labeling experiments above was not sufficiently labeled by 4 h to enable its hydrolysis product, namely DG, to be detected. Therefore, long term incubations of HeLa cells were done with [2-^{3}H]glycerol to label PC to equibilibrium. Fig. 25 shows the distribution of label in various lipids. PC was labelled to equilibrium after approximately 20 h (panel A). The labeling of various other lipids is shown in panels B and C. Table 2 shows the labeling of different lipids after 24 h as a % of the radioactivity in the total lipid fraction. Pulse-chase experiments were carried out in which cells were prelabeled for 24 h with [2-^{3}H]glycerol and then incubated ± 100 nM TPA in the absence of the label. The radiolabel in DG was determined after various times of TPA-treatment. Fig. 26 A shows that within 30 min the label in DG from control cells had decreased from 5.72 to 3.2 x 10^4 dpm/mg but in TPA-treated cells the labeled DG, after a small increase to 6.25 x 10^4 dpm/mg, remained elevated over the 2 h time period of the chase. There appeared to be no significant difference in either the label in PC (panel B) or the label in PE (panel C) in

TPA-treated or control cells. Similar pulse-chase experiments were conducted in which treatment of cells with 100 nM TPA for 1 h caused an approximately 2-fold accumulation of labeled DG compared to untreated controls (Fig. 27).

3.2.2. *Increase in Cellular Diacylglycerol Mass by TPA*

To determine whether the lack of decrease of labeled DG was due to the actual increase in DG content and not merely due to altered turnover, DG mass measurements were carried out in cells treated with TPA. Fig. 28 shows that TPA-treatment caused the DG content of cells to increase by approximately 50% within 15 min when compared to untreated controls. The colorimetric method used to measure DG mass in these experiments was, however, complicated and inconsistent with respect to absolute values between experiments. Another method was employed after DG kinase became commercially available. This method utilizes the quantitative conversion of DG to ^{32}P-labeled phosphatidic acid by DG kinase in the presence of [γ-^{32}P]ATP and the subsequent measurement of the labeled product. It is a sensitive method and gave reproducible results. Using the DG kinase method for DG quantitation it was shown that the DG mass increased from 2.29 nmol/mg in control cells to 4.02 $\pm$ 0.7 nmol/mg ($p \leq 0.0005$) in cells that had been treated with 100 nM TPA for 1 h (Fig. 29).

DG mass was also measured in microsomes prepared from HeLa cells treated $\pm$ 100 nM TPA for 1 h. Lipids were extracted and DG assayed. The DG content was found to increase from 2.77 to 6.84 nmol/mg in microsomes from cells incubated in the absence or presence of TPA, respectively.

Fig. 25. Labeling Cellular Lipids with [2-^{3}H]glycerol. HeLa cells in 100 mm dishes were incubated in the presence of 2 μCi/ml [2-^{3}H] glycerol. Cells were harvested at various times and lipids extracted. Phospholipids were separated by TLC in chloroform : methanol : acetic acid : formic acid (70 : 30 : 12 : 4 : 2). Neutral lipids were separated by TLC in diethyl ether : hexane : acetic acid (35 : 65 : 2). The separated lipids were visualized with iodine, scraped into 0.5 ml of water and subjected to liquid scintillation counting.

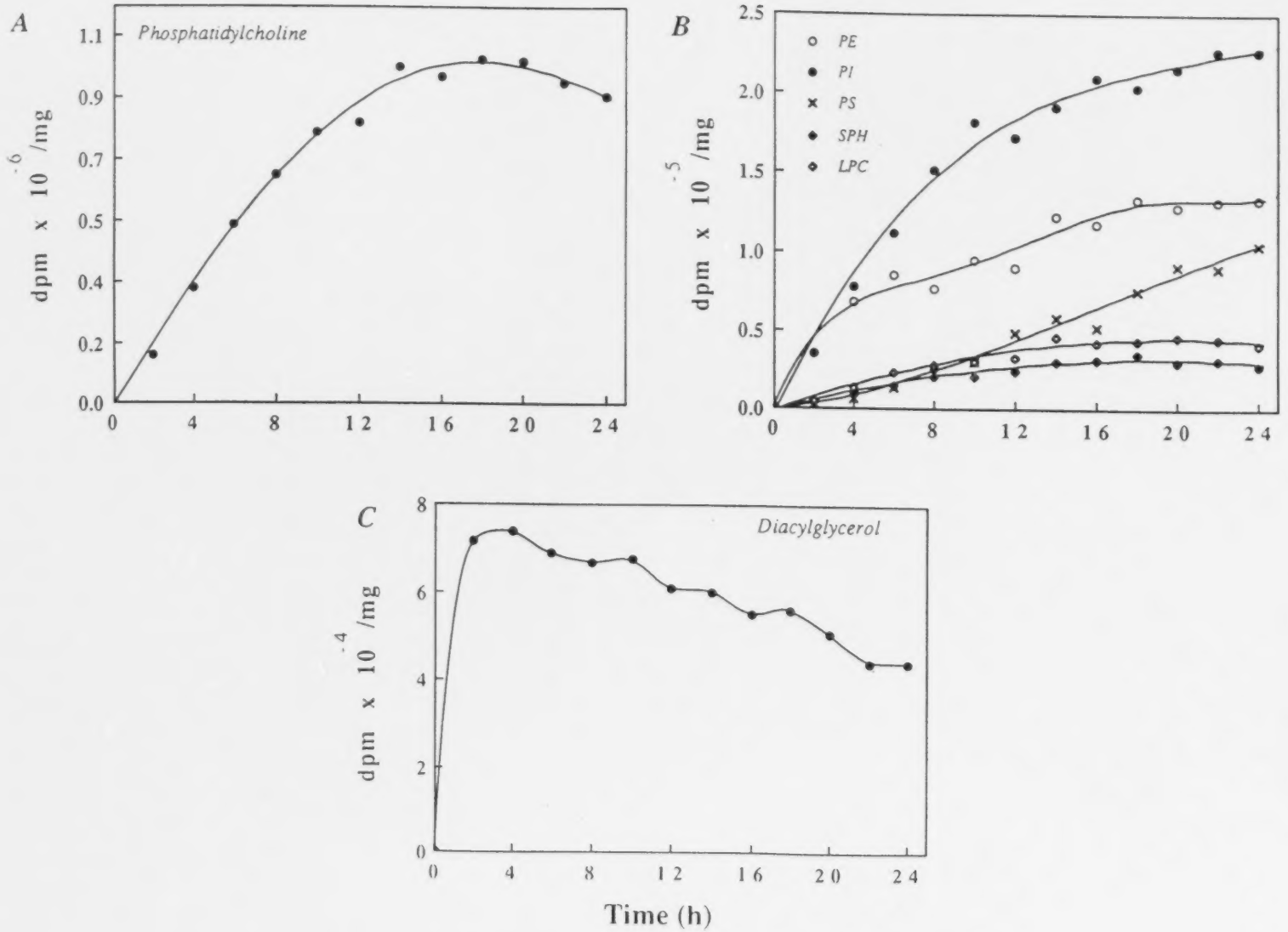

A
Phosphatidylcholine
dpm x 10 -6 /mg
1.1
0.9
0.7
0.5
0.4
0.2
0.0
0 4 8 12 16 20 24
B
PE
PI
PS
SPH
LPC
dpm x 10 -5 /mg
2.5
2.0
1.5
1.0
0.5
0.0
0 4 8 12 16 20 24
C
Diacylglycerol
dpm x 10 -4 /mg
8
6
4
2
0
0 4 8 12 16 20 24
Time (h)

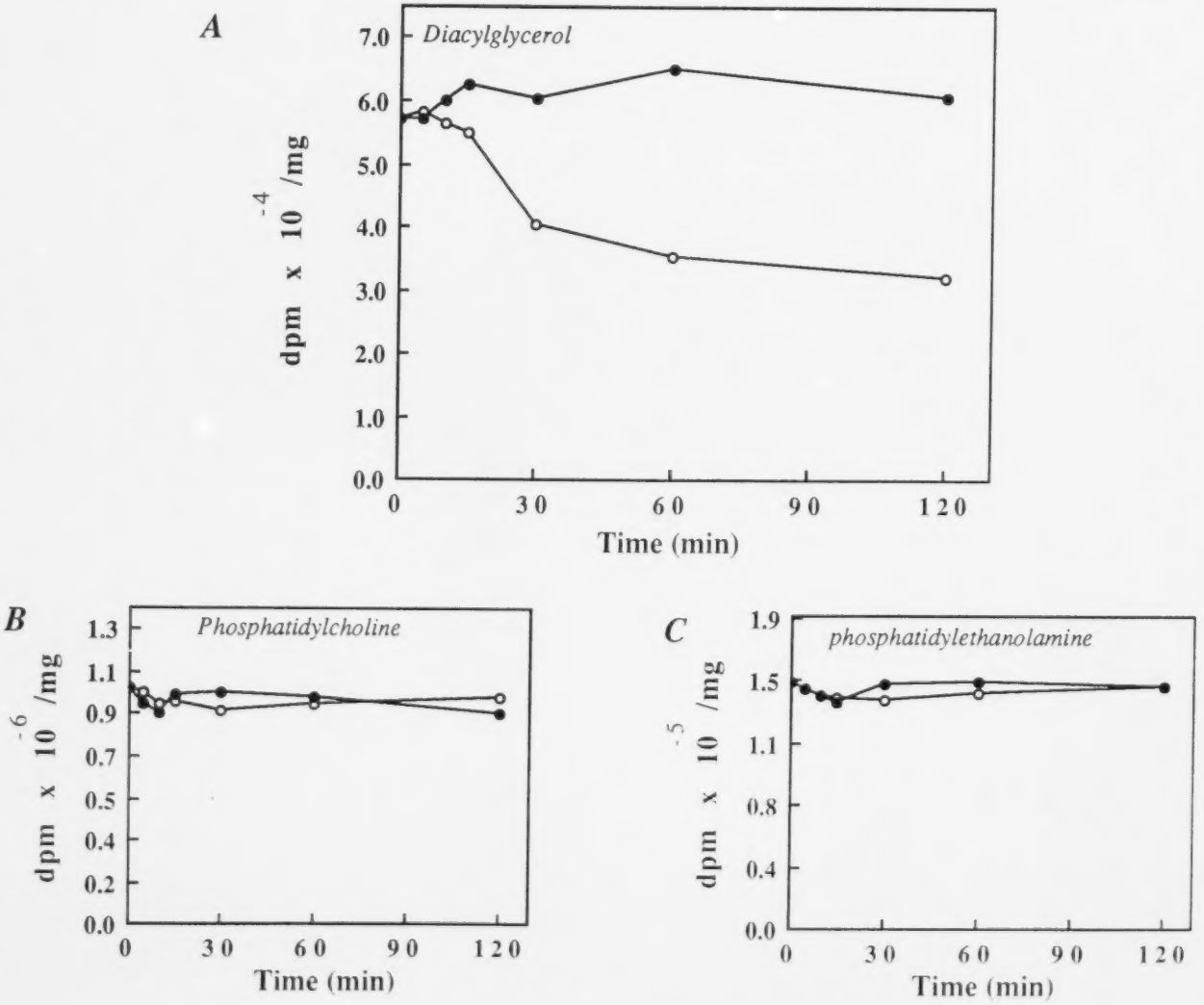
Table 2. Distribution of [2-^{3}H]glycerol in Different Lipids.

HeLa cells in 100 mm dishes were incubated in the presence of 2 μCi/ml of [2-^{3}H]glycerol. Cells were harvested after 24 h and lipids extracted. Phospholipids were separated by TLC in chloroform : methanol : acetic acid : formic acid : water (70 : 30 : 12 : 4 : 2). Neutral lipids were separated by TLC in diethyl ether : hexane : acetic acid (35 : 65 : 2). The separated lipids were visualized with iodine, scraped and subjected to liquid scintillation counting.

	dpm (as % of total)[a]
phosphatidylcholine	65.0
phosphatidylethanolamine	7.0
phosphatidylinositol	13.1
phosphatidylserine	6.0
sphingomyelin	1.8
lysophosphatidylcholine	2.7
diacylglycerol	4.4

a The results are expressed as % of the radioactivity in the total lipid fraction

Fig. 26. Effect of TPA on the Levels of [^{3}H]DG, PC and PE. HeLa cells were prelabeled with 2 μCi/ml of [2-^{3}H]glycerol for 24 h. The labeled medium was removed, the cells rinsed thrice and incubated in the presence of 0.1% DMSO (open symbols) or 100 nM TPA (solid symbols). At various times cells were harvested, lipids extracted and separated by TLC. Lipids were visualized with iodine, scraped into 0.5 ml water and subjected to liquid scintillation counting. The radioactivity in DG (Panel A), PC (Panel B) and PE (Panel C) was determined. The data are means of duplicate samples and representative of three separate experiments.


A
Diacylglycerol
dpm x 10 /mg
-4
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
0
30
60
90
120
Time (min)
B
Phosphatidylcholine
dpm x 10 /mg
-6
1.3
1.1
0.9
0.7
0.5
0.4
0.2
0.0
0
30
60
90
120
Time (min)
C
phosphatidylethanolamine
dpm x 10 /mg
-5
1.9
1.5
1.1
0.8
0.4
0.0
0
30
60
90
120
Time (min)

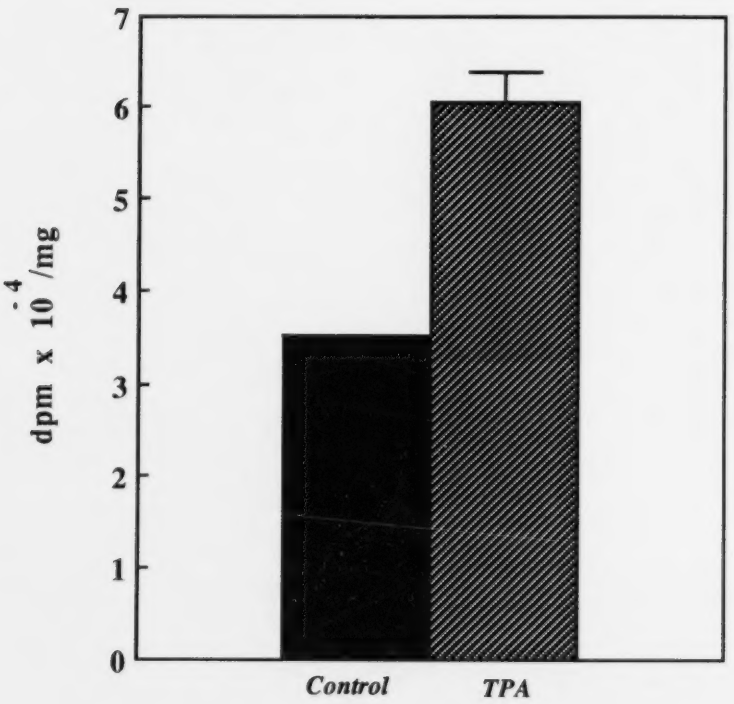


Fig. 27. Accumulation of [^{3}H]DG in TPA-Treated Cells.

HeLa cells were prelabeled for 24 h in the presence of 2μCi/ml [2-^{3}H]glycerol. Subsequently, the labeled medium was removed, the cells rinsed thrice and incubated in the presence of 0.1% DMSO (*Control*) or 100 nM *TPA*. After 1 h cells were harvested, lipids extracted and separated by TLC in diethyl ether : hexane : acetic acid (35 : 65 : 2). The radioactivity in DG was determined by liquid scintillation counting and expressed on a per mg protein basis. The data (mean ± S.D.) are from four determinations and plotted after normalization to control values.

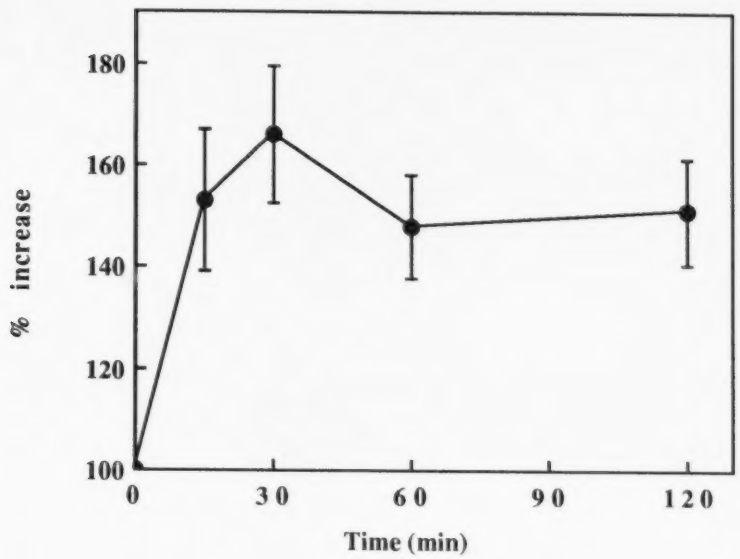


Fig. 28. DG Accumulation in TPA-Treated Cells. HeLa cells were incubated ± 100 nM TPA. At various times cells were harvested, DG extracted and assayed using enzymatic analysis as described in 'Experimental Procedures'. The results ± are expressed as % increase of DG over control. The average control value was 9.9 ± 3.4 nmol/mg protein (n=5).

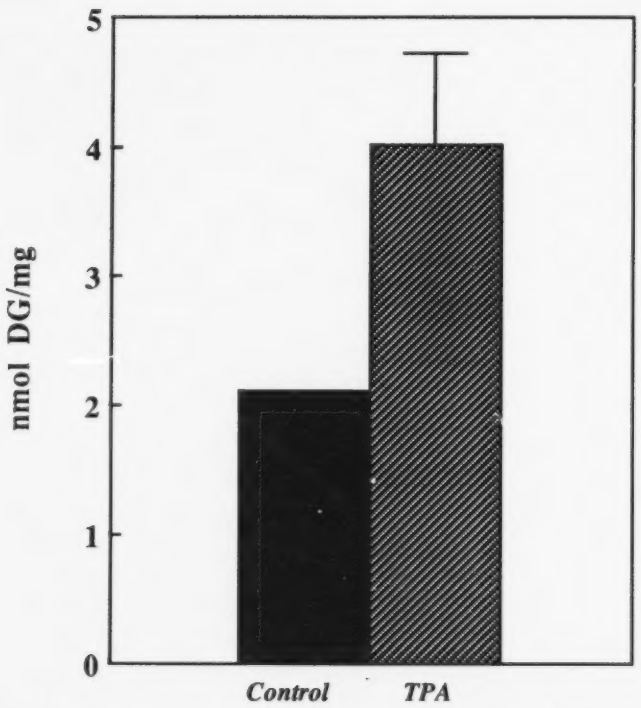


Fig. 29. Accumulation of DG Mass in TPA-Treated Cells. HeLa cells were incubated in medium containing 0.1% DMSO (Control) or 100 nM TPA for 1 h, followed by lipid extraction and DG quantitation by the DG Kinase method. The data (mean ± S.D.) are from four determinations expressed on a per mg protein basis and plotted after normalization to control values.

3.2.3. *TPA-Mediated DG Increase as a Function of Time*

The time-course of DG accumulation was investigated (Fig. 30). HeLa cells were incubated ± 100 nM TPA and the cells harvested at various times. Lipids were extracted and the DG levels determined. An increase in DG mass was observed from 1.7 at zero time to 2.5 nmol/mg after 10 min of TPA-treatment. The increase in DG mass was maximal by 15 min at 4.31 nmol/mg.

3.3. FURTHER EVALUATION OF DG AS A TRANSLOCATION SIGNAL FOR CT

In section 3.2 it was demonstrated by different methods that DG accumulated in cells treated with TPA. Treatment of HeLa cells with TPA has been shown to generate DG by the hydrolysis of PC via a protein kinase C (PKC) activated pathway (Besterman *et al.*, 1986; Daniel *et al.*, 1986; Glatz *et al.*, 1987). It was, therefore, possible that the translocation of CT to membranes observed upon TPA-treatment was due to a decrease in the cellular PC content rather than the increase in DG content. There is evidence that the amount of membrane-associated CT activity can be influenced by the membrane PC levels (Jamil *et al.*, 1990). To dissociate these two events, i.e., increased cellular DG and PC hydrolysis, it was proposed that cellular DG levels be increased by incubating the cells in the presence of an exogenous source of DG such as DiC_8, a cell-permeant analogue of DG. This would increase cellular DG levels without affecting PC hydrolysis. However, DiC_8 is known to activate PKC (Lapetina *et al.*, 1985) and since the activation of PKC would result in PC hydrolysis, a modified approach was required so that PKC would be bypassed.

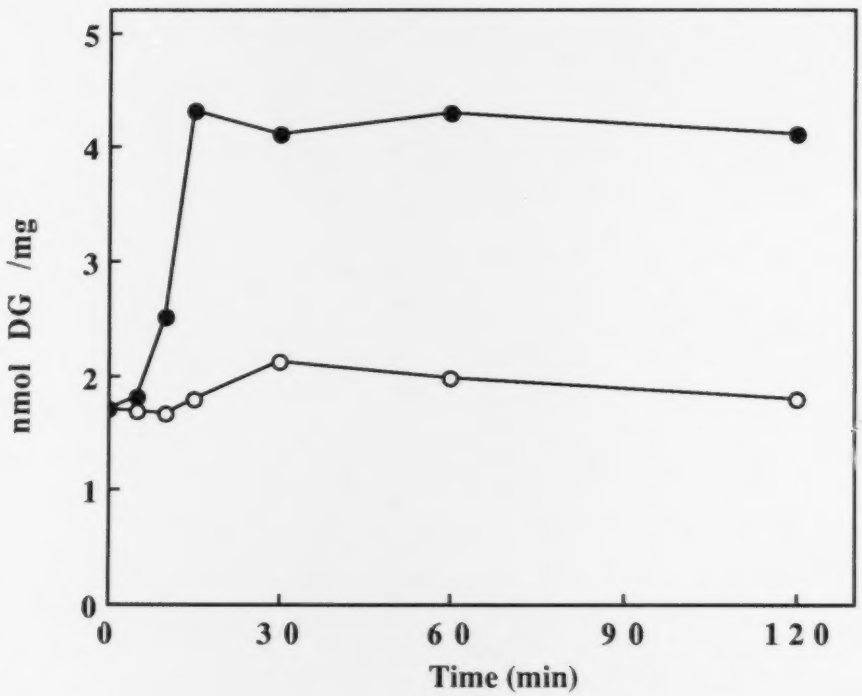


Fig. 30. Time-Course of DG Accumulation in TPA-Treated Cells.

Confluent HeLa cells were incubated in medium containing 0.1% DMSO (open symbols) or TPA (solid symbols). At various times cells were harvested, lipids extracted and DG quantitated. The data are expressed on a per mg protein basis, are means of duplicate samples and representative of three separate experiments.

3.3.1 Downregulation of Protein Kinase C

Prolonged incubation of various cell types with TPA has been shown to result in the depletion of PKC from the cells (Rodriguez-Pena and Rozengurt, 1984; Ballester and Rosen, 1985). This phenomenon is known as down-regulation. Diacylglycerol or phorbol ester mediated translocation of PKC to the plasma membrane makes PKC susceptible to proteolysis and prolonged treatment results in its complete disappearence as demonstrated by immunological studies (Ballester and Rosen, 1985). HeLa cells were downregulated for PKC by incubating them in the presence of 1 μM TPA. At various intervals cells were harvested and fractionated into cytosolic and particulate fractions. The proteins in the samples were separated by SDS-PAGE and subjected to immunoblot analysis using a commercially available monoclonal antibody to PKC. This antibody recognizes the hinge region between the catalytic and the regulatory domains of the α and β (βI and βII) isoforms of PKC. HeLa cells have been reported to have predominantly the α isoform (Hii *et al.*, 1990). Fig. 31 shows the results of an immunoblot analysis using ^{125}I-protein A for PKC visualization. Within 15 min all the immunologically detectable PKC had translocated from the cytosol to the membranes and by 22 h it had all disappeared. In another experiment (not shown) twice the amount of particulate protein from the 22 h sample was included for immunoblot analysis and still no PKC band was visible, further indicating that the cells were depleted of PKC.

Another more sensitive method involving the use of chemiluminescence for the detection of PKC on immunoblots was utilized. The principle of the method is illustrated in Appendix B and described in section 2.19.2. Fig. 32 shows the results of an autoradiogram obtained after a 5 second

exposure. A longer exposure of 15 seconds (panel B) failed to detect any PKC at 22 h or 24 h of TPA-treatment.

Thus, the results of the immunoblot analyses indicate that by 22 h the cells had been downregulated for PKC.

3.3.2 *Translocation of CT and Stimulation of PC Biosynthesis by Exogenous Diacylglycerols*

Downregulated HeLa cells were incubated in the presence of sonicated suspensions of 0.5 mM of three different types of diacylgylcerols; DiC_8, OaG (1-Oleoyl-2-acetyl-*sn*-glycerol) and egg PC-derived DG (made by the *C. welchii* hydrolysis of egg PC as described in section 2.15.2 a). After 30 min membrane-associated CT activity from digitonin-permeabilized cells was determined (Fig. 33). DiC_8 caused the maximal CT translocation from 0.18 in untreated to 0.30 nmol/min/mg in DiC_8-treated cells (lane 2), while egg PC-derived DG was without effect (lane 4), presumably due to its inability to enter cells because of its highly hydrophobic nature. Parallel pulse-chase experiments (Fig. 34) with all three DG's revealed that the CT translocation elicited by them was functional as seen by resulting stimulation of PC biosynthesis. Panel B shows that DiC_8 treatment caused the incorporation of [*methyl*-^{3}H]choline into the total lipid fraction to increase from 1.3 (lane 1) to 2.71 x 10^5 dpm/mg (lane 2). OaG caused a lesser increase to 1.92 x 10^5 dpm/mg (lane 3), while egg PC-derived DG was without effect (lane 4). The radiolabel in the aqueous choline metabolites (panel A) decreased concomitantly, indicating increased formation of CDP-choline i.e., increased activity of CT. These results, therefore, agree with a DG-stimulated CT activity. Since DiC_8 gave the maximum response of all three DG's it was chosen to be the exogenous source of DG in all subsequent experiments.

Fig. 31. Immunoblotting of PKC. 100 µg of cytosolic and particulate proteins from HeLa cells treated with 1 µM TPA for various times were separated by SDS-PAGE and subjected to immunoblot analysis using a commercially available monoclonal antibody to PKC (MC 5) and visualized with ^{125}I-Protein A. Autoradiographs were obtained after exposing the immunoblots to X-ray film in a film cassette with intensifier screens for 5 days at -70°C.

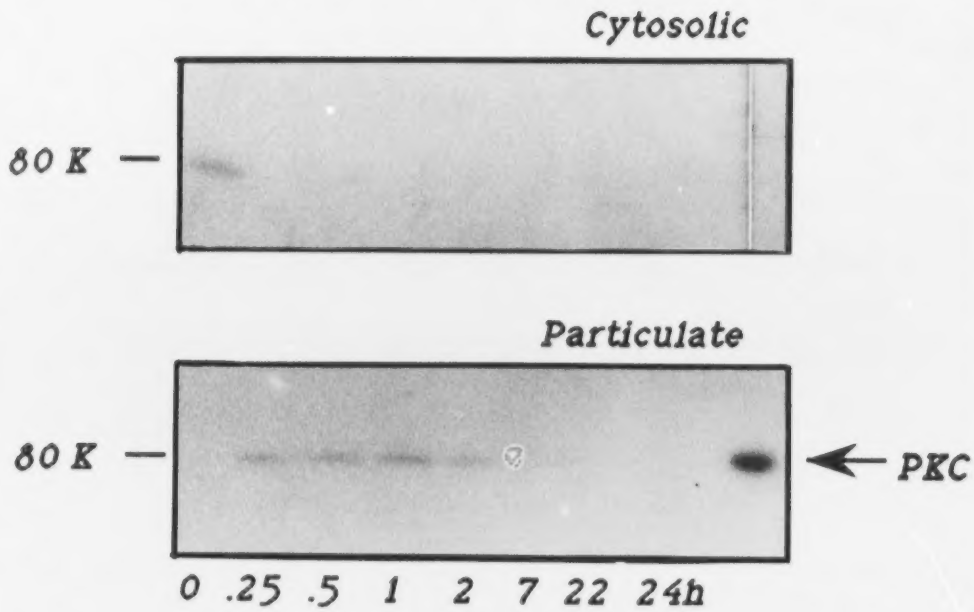
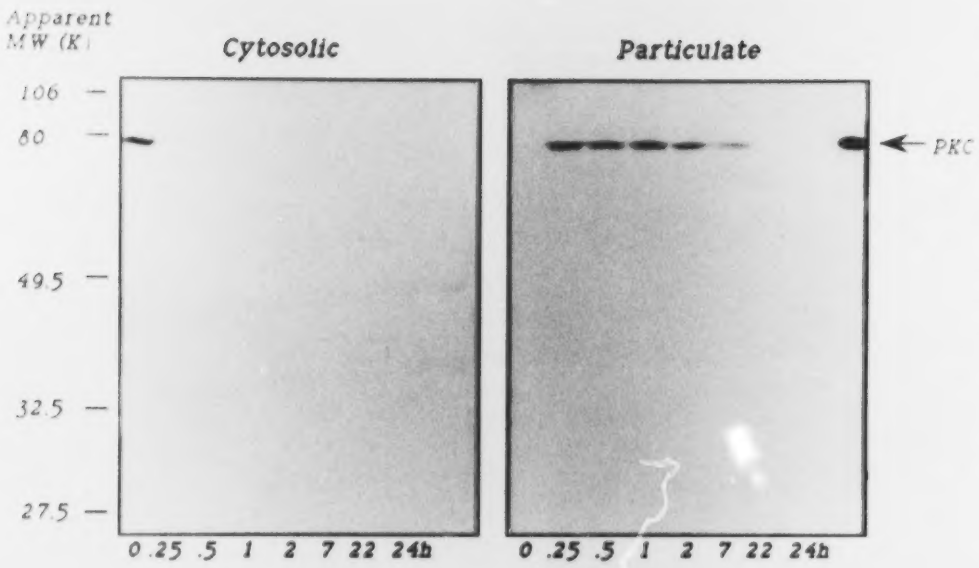
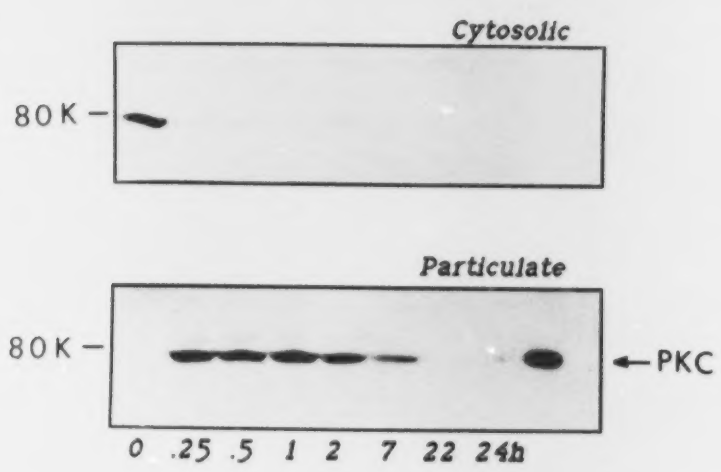

Cytosolic
80 K
Particulate
80 K
PKC
0 .25 .5 1 2 7 22 24h

Fig. 32. Downregulation of PKC as Visualized by Immunoblotting. 100 µg of cytosolic and particulate proteins from HeLa cells treated with 1 µM TPA for various times were separated by SDS-PAGE and subjected to immunoblot analysis using a commercially available monoclonal antibody to PKC (MC 5) and a chemiluminecsent method for visualizing antigen. Panel A shows autoradiographs after 5 seconds exposure of the blots to X-ray film, whereas Panel B shows results after 15 seconds exposure. The 80 kD band in the right-hand lane of the membrane blots shows pure bovine brain PKC.

A


Apparent
MW (K)
Cytosolic
Particulate
106
80
49.5
32.5
27.5
PKC
0 .25 .5 1 2 7 22 24h
0 .25 .5 1 2 7 22 24h

B


Cytosolic
80K
Particulate
80K
PKC
0 .25 .5 1 2 7 22 24h

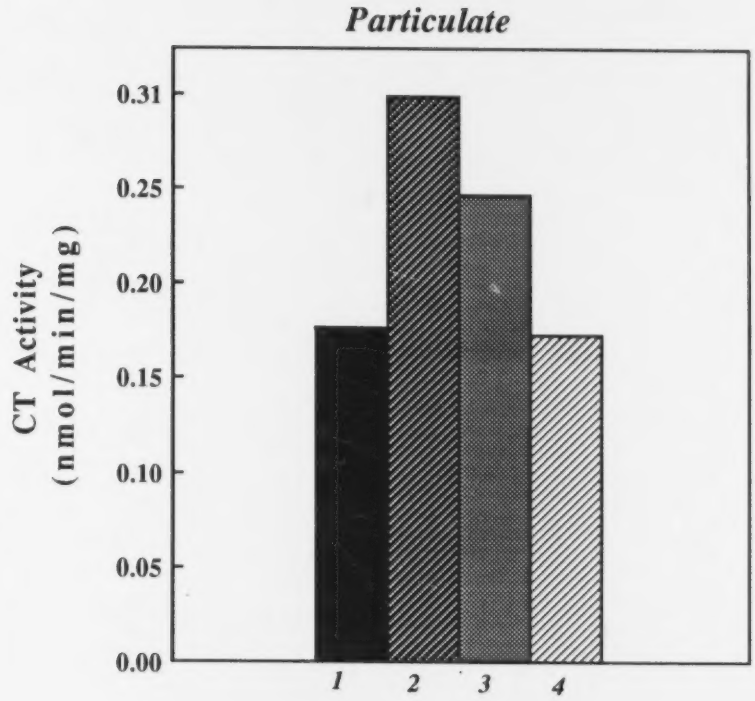
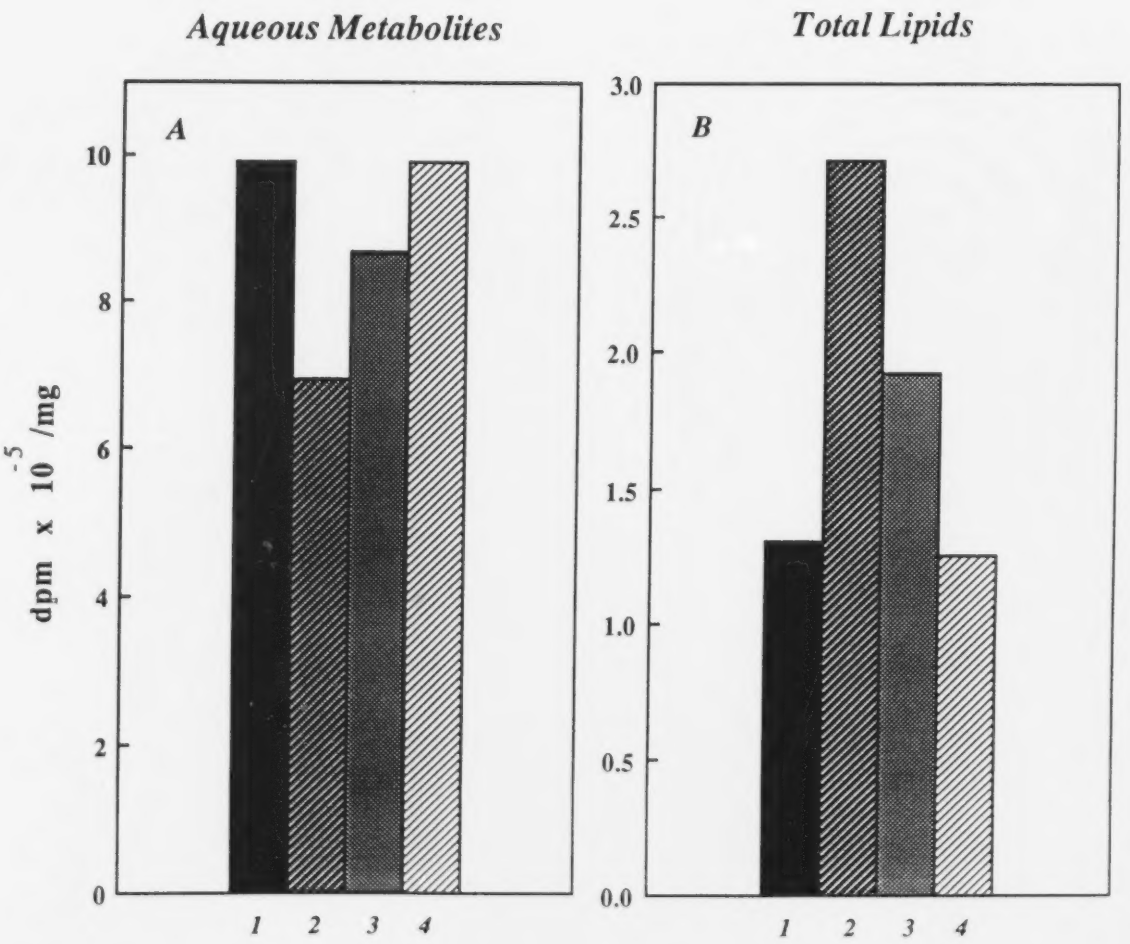


Fig. 33. Translocation of CT by Various Diacylglycerols. PKC-downregulated HeLa cells were incubated in the absence (lane *1*) or presence of 0.5 mM DiC_8 (lane *2*), OaG (lane *3*) and egg PC-derived DG (lane *4*). After 30 min cells were digitonin-permeabilized, membrane CT activities were determined and expressed as per mg protein.

Fig. 34. Stimulation of PC Biosynthesis by Various Diacylglycerols. PKC-downregulated HeLa cells were prelabeled with 2 μCi/ml [*methyl*-^{3}H]choline chloride for 1 h. Subsequently, the labeled medium was removed, the cells rinsed thrice and chased in the absence (lane *1*) or presence of 0.5 mM DiC_8 (lane *2*), OaG (lane *3*) and egg PC-derived DG (lane *4*). After 1 h the cells were harvested, lipids extracted and the radioactivity in the choline-containing aqueous metabolites (Panel *A*) and the total lipid fraction (Panel *B*) determined.


Aqueous Metabolites
Total Lipids
A
B
dpm x 10 -5 /mg
0
2
4
6
8
10
0.0
0.5
1.0
1.5
2.0
2.5
3.0
1 2 3 4
1 2 3 4

3.3.3. *DiC_8 Stimulates CT Translocation and PC Biosynthesis in PKC-Downregulated HeLa Cells*

The effect of DiC_8 on CT translocation and PC biosynthesis was examined with an additional control which excluded TPA from incubations ± DiC_8 after down-regulation. In these experiments 100 nM TPA was included in all the incubations subsequent to PKC-downregulation. It was possible that the presence of TPA enhanced the DG-stimulated effects. Therefore, a control was included where TPA was excluded after PKC-downregulation (Figs. 35 and 36). The results indicate that the effects observed were independent of the presence or absence of TPA in the media during incubations ± DiC_8. PKC-downregulated cells were incubated for 30 min in medium alone, medium containing 100 nM TPA or medium containing 100 nM TPA plus 0.5 mM DiC_8. Particulate CT activity increased to 0.67 ± 0.02 nmol/min/mg in DiC_8-treated cells from 0.41 ± 0.07 and 0.37 ± 0.04 nmol/min /mg in controls (cells incubated in medium alone and medium containing 100 nM TPA, respectively) as seen in Fig. 35. This translocation would be expected to enhance PC biosynthesis. Therefore, PKC-downregulated cells were prelabeled with [^{3}H]choline for 1 h and incubated for a further 1 h in unlabeled medium alone, medium containing 100 nM TPA or medium containing 100 nM TPA plus 0.5 mM DiC8 (Fig. 36). DiC_8 stimulated the incorporation of labeled aqueous metabolites into the total lipid fraction (as seen by the decrease in label in the aqueous metabolites and the increase in label in the organic phase). The label in total lipids increased from 1.54 ± 0.06 and 1.56 ± 0.10 x 10^5 dpm/mg in controls (lanes 1 and 2) to 2.95 ± 0.30 x 10^5 dpm/mg in DiC_8-treated cells (lane 3).

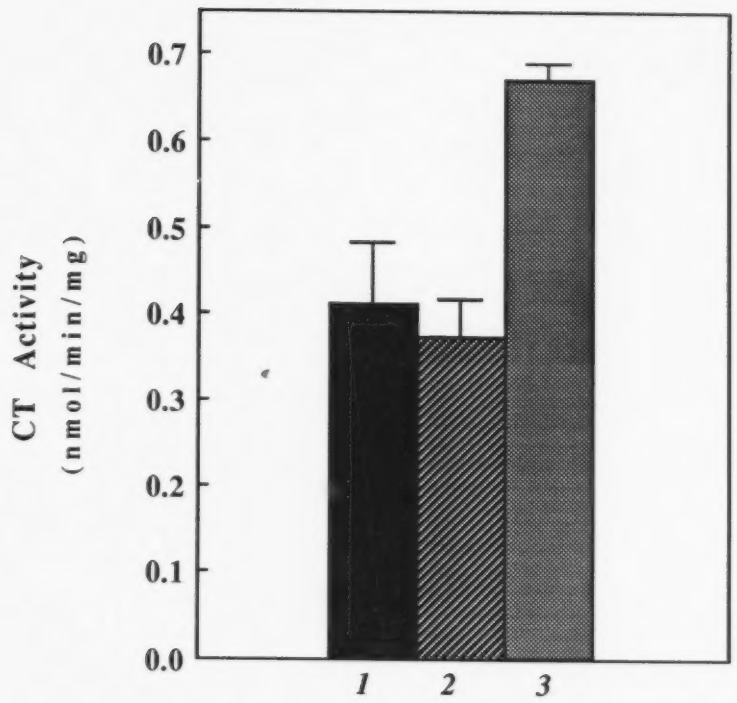
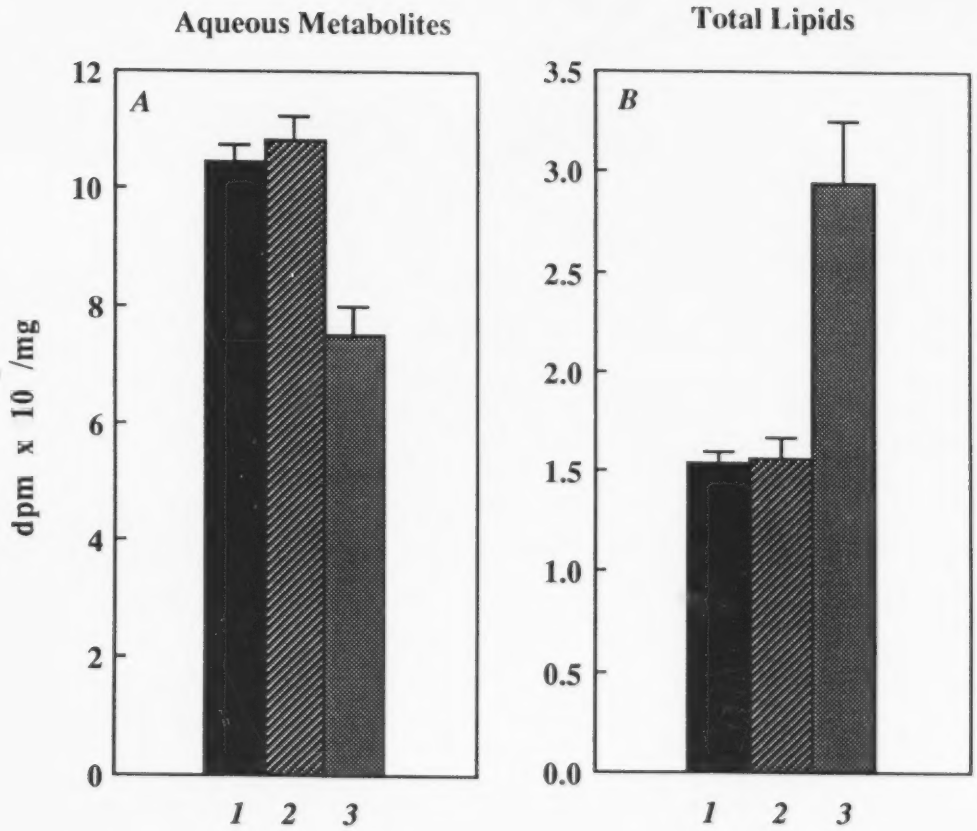


Fig. 35. Effect of DiC_8 on CT Translocation in PKC-Downregulated Cells. HeLa cells were incubated for 22-24 h in growth medium containing 1 μM TPA. The medium was removed and cells were incubated for 1 h in medium without TPA (*1*), with 100 nM TPA (*2*) or with 100 nM TPA plus 0.5 mM DiC_8(*3*). The medium was removed, cells were digitonin-permeabilized for 5 min and particulate CT activity was determined. The data (mean ± S.D.) represent duplicate values from three separate experiments and are expressed on a per mg protein basis.

Fig. 36. Effect of DiC_8 on PC Biosynthesis in PKC-Downregulated Cells. HeLa cells in 60 mm dishes were incubated for 22-24 h in growth medium containing 1 μM TPA. This medium was removed and the cells prelabeled for 1 h with medium containing 100 nM TPA and 2 μCi/ml [^{3}H]choline chloride (4 μCi/dish). The labeled medium was removed, and the cells were incubated for a further 1 h in medium without TPA (*1*), with 100 nM TPA (*2*) or with 100 nM TPA, 0.5 mM DiC_8 (*3*). Cells were harvested, lipids were extracted and the radiolabel in aqueous phase and organic phase determined. The data (mean ± S.D.) are duplicate determinations from three separate experiments.

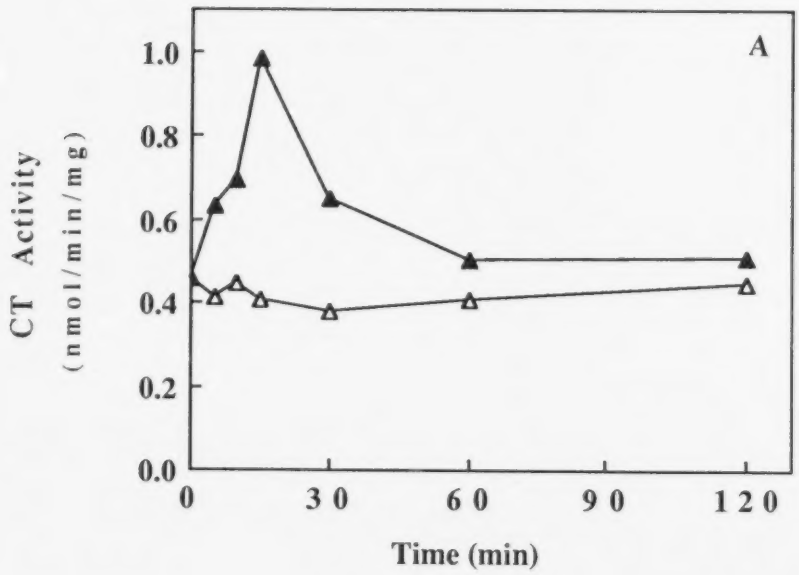

Aqueous Metabolites
Total Lipids
A
B
dpm x 10 -5 /mg
12
10
8
6
4
2
0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
1 2 3
1 2 3

3.3.4. *Time-Course of DiC_8-Elicited CT Translocation and PC Biosynthesis*

The DiC_8-induced CT translocation was examined as a function of time (Fig. 37 A). PKC-down regulated cells were incubated in the absence or presence of 0.5 mM DiC_8 and the translocation of CT to membranes examined at different times. Within 10 min of DiC_8 treatment CT activity in membranes had increased from 0.46 to 0.63 nmol/min/mg and to 0.90 nmol/min/mg at 15 min. A surprising finding was that by 30 min particulate CT activity in DiC_8-treated cells had returned to 0.65 nmol/min/mg and continued to decrease so that by 1 h and 2 h, CT activity was near control levels.

The time course of DiC_8-induced PC biosynthesis was also examined (Fig. 37 B) in pulse-chase experiments using PKC-downregulated cells. The incorporation of [^{3}H]choline into total lipids was examined at various times up to 120 min. After a short (5 min) lag increased incorporation of radiolabel into lipids was seen in the DiC_8-treated cells. This accelerated over 15 min and the rate of incorporation then slowed to near control levels. Fig. 38 A shows a plot of the rate of [^{3}H]choline incorporation into total lipids at a specific time point calculated with respect to the previous time point using data generated from Fig. 37 B. At 5 min the rate was the same as that of controls, but between 5 and 10 min the rate increased to 6,936 dpm/min and was maximal between 10 and 15 min (8,101 dpm/min). In agreement with the profile of DiC_8-induced translocation of enzyme, the rate at 30 min decreased to 3,552 dpm/min and at 1 h and 2 h approached the control levels of approximately 1,500 dpm/min. A plot of the rate of incorporation of label into total lipids against particulate CT activity showed a positive correlation (r^2=0.89, Fig. 38***B***).

Fig. 37. Time Course of DiC_8-Elicited CT Translocation(A) and PC Biosynthesis(B) in PKC-Downregulated Cells. HeLa cells were incubated for 22-24 h in growth medium containing 1 μM TPA. **(A)**The medium was removed and cells were incubated for the indicated times in medium containing 100 nM TPA (open symbols) or 100 nM TPA, 0.5 mM DiC_8 (solid symbols). The cells were digitonin-permeabilized for 5 min and particulate CT activity was determined. **(B)**The medium was removed and cells were incubated in medium containing 100 nM TPA and 2 μCi/ml [^{3}H]choline chloride (4 μCi/dish). After 1 h the medium was removed, cells were rinsed thrice and were incubated for the indicated times in chase medium containing 100 nM TPA (open symbols) or 100 nM TPA, 0.5 mM DiC_8 (solid symbols). The data are means of duplicates from one of 3 similar experiments.


A
CT Activity
(nmol/min/mg)
1.0
0.8
0.6
0.4
0.2
0.0
0
30
60
90
120
Time (min)

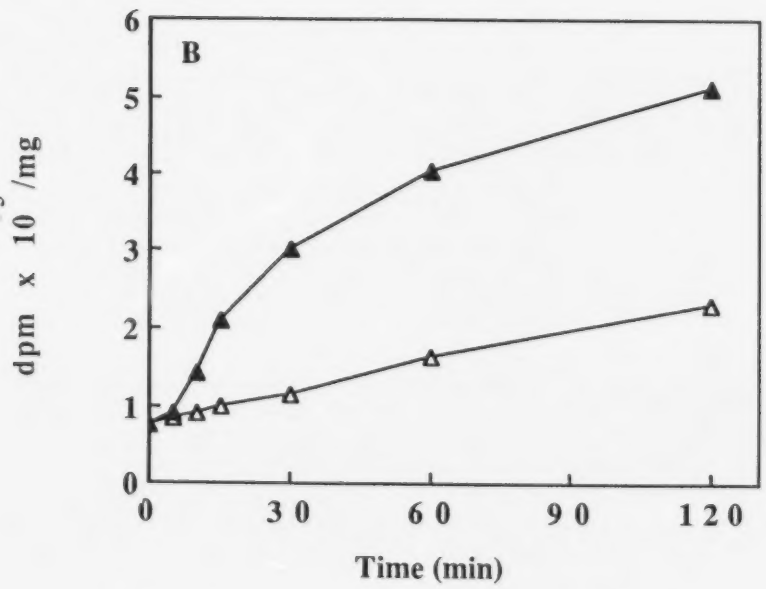
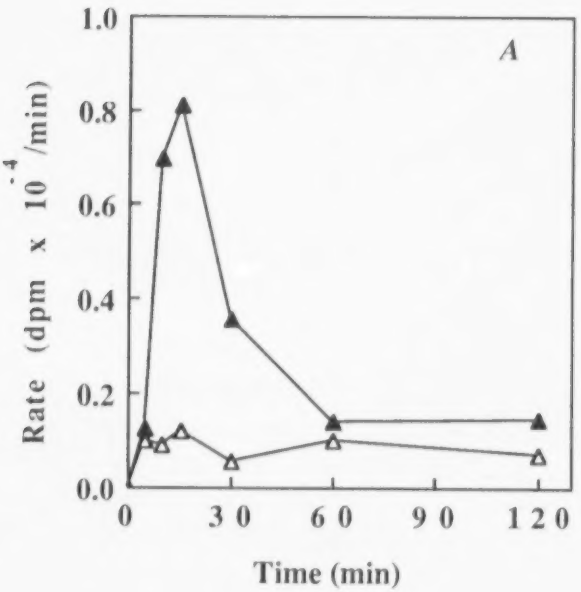
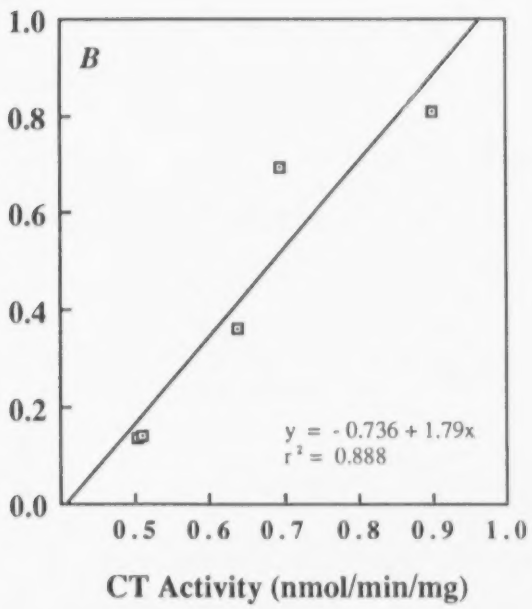

B
dpm x 10 -5 /mg
6
5
4
3
2
1
0
0
30
60
90
120
Time (min)

Fig. 38. Plot of Rate of PC Biosynthesis and Correlation With Particulate CT Activity. Panel *A*, The rates of [^{3}H]choline incorporation into total lipids induced by DiC_8 between two consecutive time points were calculated (data of Fig. 37B) and plotted against the later time. Panel *B*, the calculated rates in panel *A* were plotted against particulate CT activities (data from Fig. 37A) and a correlation obtained.


A
1.0
0.8
0.6
0.4
0.2
0.0
Rate (dpm x 10 -4 /min)
0
30
60
90
120
Time (min)


B
1.0
0.8
0.6
0.4
0.2
0.0
y = - 0.736 + 1.79x
r 2 = 0.888
0.5
0.6
0.7
0.8
0.9
1.0
CT Activity (nmol/min/mg)

3.3.5. *Confirmation of DiC_8-Elicited CT Translocation by Immunoblot Analysis*

The translocation of CT activity to membranes after DiC_8-treatment of PKC-downregulated cells was examined further by immunoblot analysis. HeLa cells were downregulated for 22-24 h in the presence of 1 μM TPA and treated with 0.5 mM DiC_8 for 15 min (incubation with DiC_8 was only for 15 min because CT translocation was maximal at that time, Fig. 35). The proteins in a solubilized particulate fraction were separated by SDS-PAGE and subjected to immunoblot analysis (Fig. 39). The results show that the increase in membrane-associated CT activity seen in DiC_8-treated cells is due to an increase in the amount of the enzyme associated with the membranes.

3.3.6. *DiC_8 Accumulation in DiC_8-Treated Cells*

To attribute the increase in membrane-associated CT in DiC_8-treated cells to increased levels of DG, it was necessary to show that cellular DG levels had indeed increased. Fig. 40 shows the levels of DG at various times of DiC_8-treatment of PKC-downregulated cells. DiC_8 mass increased maximally to 2.75 nmol/mg within 10 min and then decreased to 1.24 nmol/mg after 2 h of DiC_8-treatment (panel A). The levels of long-chain DG were also measured and no significant difference was found between untreated and DiC_8-treated cells.

It was also imperative to show that membrane-associated DG levels were elevated in DiC_8-treated cells to make their role as signals for CT translocation more significant. Therefore, membrane-associated DiC_8 mass was quantitated in the particulate fraction of digitonin-permeabilized cells which had been treated with 0.5 mM DiC_8 for 15 min after PKC-downregulation and found to be 5.18 ± 1.54 nmol/mg. Again long-chain DG levels were found to be unchanged (6.15 ± 0.34 in control compared to 7.72 ± 1.38 nmol/mg in DiC_8-treated cells).

Fig. 39. Immunoblot Analysis of DiC_8-Elicited CT Translocation.

PKC - downregulated HeLa cells in 60- and 100-mm dishes were incubated in the absence or presence of 0.5 mM DiC_8. After 15 min the cells were digitonin-permeabilized for 5 min and particulate CT activity determined in the samples from the 60-mm dishes. The particulate fractions from the 100-mm dishes were solubilized, the proteins separated by SDS-PAGE and subjected to immunoblot analysis using a rabbit antibody to CT as described in section 2.17.2. CT was visualized by enhanced chemiluminescence (Appendix B). The results of particulate CT activities in control cells (solid bar) and in DiC_8-treated cells (hatched bar) are shown in the bar graph. The corresponding insets are portions of autoradiographs obtained from the parallel immunoblot experiment showing particulate CT.

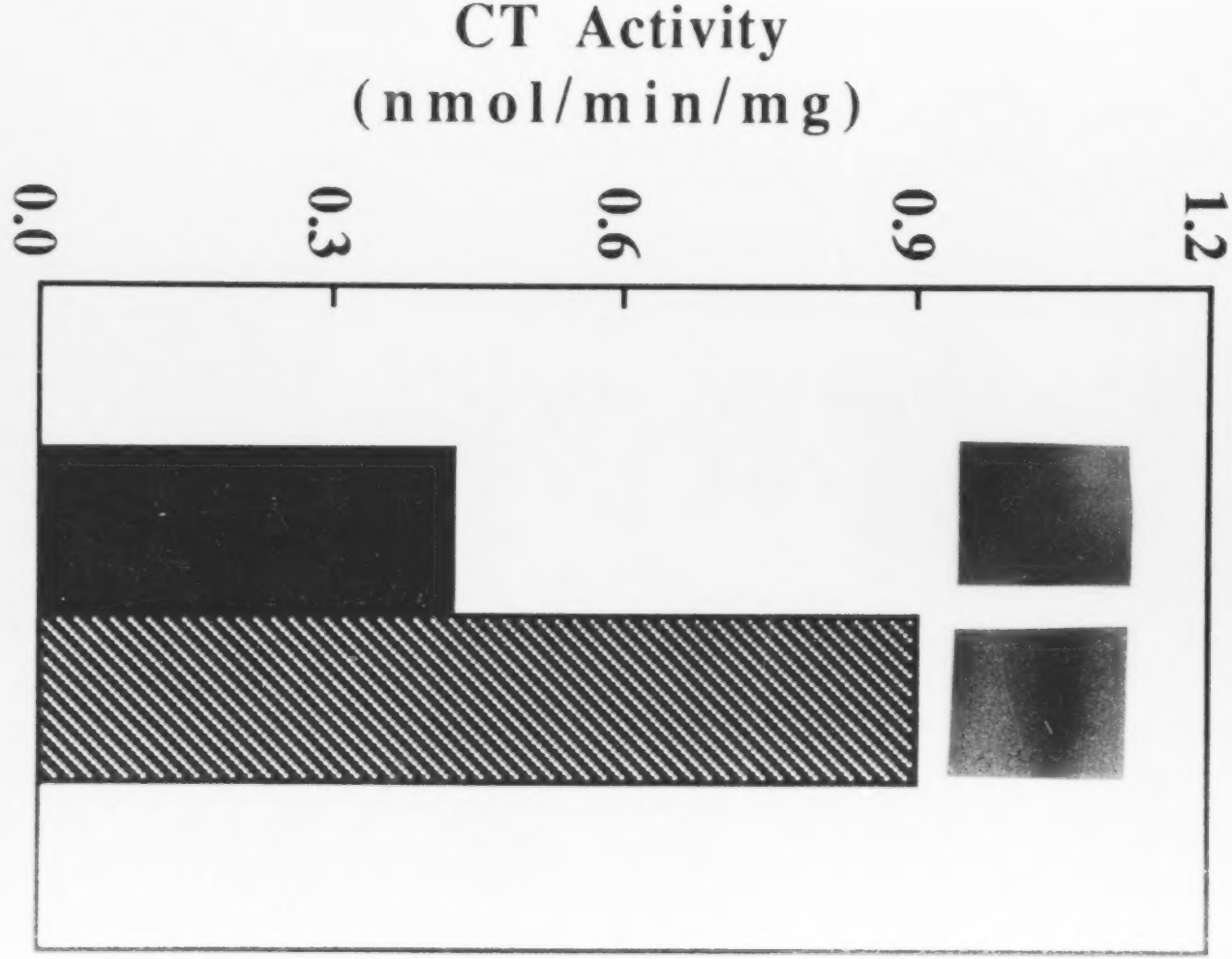
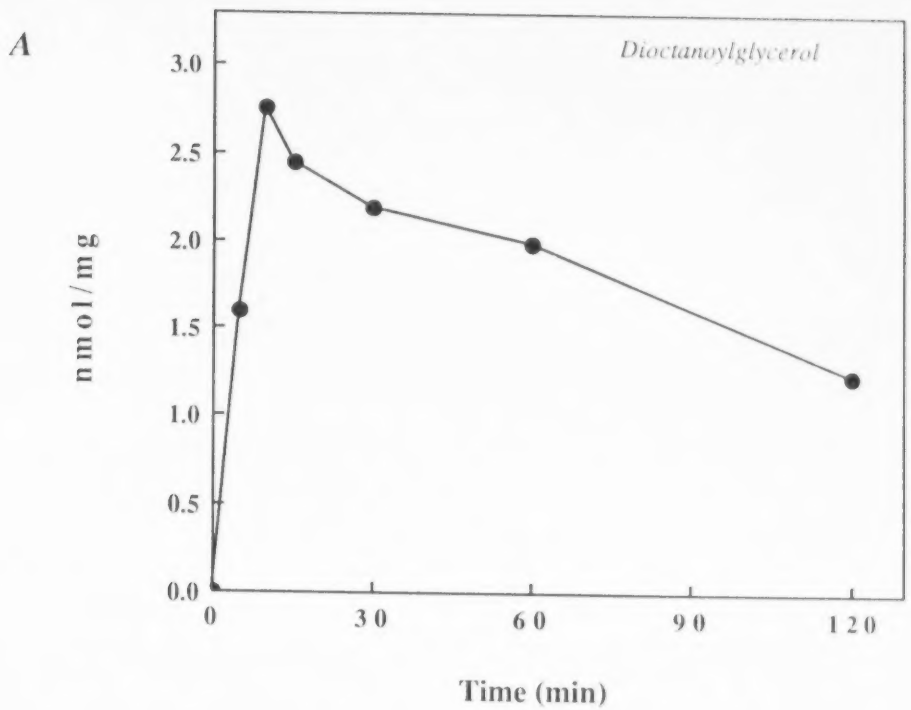
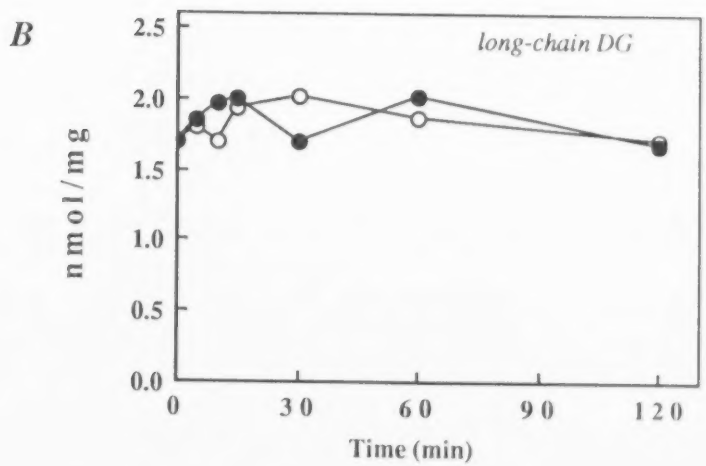

CT Activity
(nmol/min/mg)
0.0
0.3
0.6
0.9
1.2

Fig. 40. DiC_8 Accumulation in DiC_8-Treated Cells. PKC-downregulated HeLa cells were incubated in the absence (open symbols) or presence (solid symbols) of 0.5 mM DiC_8. At the indicated times cells were harvested, lipids extracted and DG quantitated by the DG kinase method. The resulting [^{32}P]phosphatidic acid was separated from dioctanoyl-[^{32}P]phosphatidic acid by TLC in chloroform : acetone : methanol : acetic acid : water (10 : 4 : 3 : 2 : 1) as described in section 2.15.2. The positions of the relevant bands were detected by autoradiography. The bands were scraped into 0.5 ml water and subjected to liquid scintillation counting. Panels A and B show the mass of DiC_8 and long chain DG, respectively.


A
Dioctanoylglycerol
nmol/mg
3.0
2.5
2.0
1.5
1.0
0.5
0.0
0
30
60
90
120
Time (min)


B
long-chain DG
nmol/mg
2.5
2.0
1.5
1.0
0.5
0.0
0
30
60
90
120
Time (min)

3.3.7. *Incorporation of DiC_8 into PC*

Since the levels of long-chain DG remained unchanged during DiC_8-treatment of PKC-downregulated cells it appeared that an alternative source of DG was being used for incorporation into PC via the cholinephosphotransferase reaction. Therefore, the incorporation of DiC_8 into PC to form dioctanoylphosphatidylcholine (DiC_8-PC) was investigated by three different methods:

(a) The formation of DiC_8-PC as seen in [^{3}H]choline pulse-chase studies - PKC-downregulated HeLa cells were prelabeled with 2 µCi/ml of [*methyl*-^{3}H]choline chloride for 1 h and the labeled medium replaced with medium ± 0.5 mM DiC_8. After various times the cells were harvested, lipids extracted and phospholipids separated by TLC. The radiolabel in long-chain PC and DiC_8-PC was determined (Fig. 41). Panel A shows that there was increased incorporation of [^{3}H]choline into long-chain PC in DiC_8-treated cells compared to controls. This increase (1.5-fold at 30 min) was insufficient to account for the more than 2-fold stimulation of [^{3}H]choline incorporation into the total lipid fraction seen in Fig. 37 B. However, the incorporation of [^{3}H]choline into DiC_8-PC could account for the rest of the stimulation of PC biosynthesis. Table 3 shows the relative distributions of radiolabel in long-chain PC and DiC_8-PC. In control cells 98% of the radioactivity in the total lipid fraction was associated with PC, whereas in DiC_8-treated cells, 60% was associated with long-chain PC and 31% with DiC_8-PC. When the results were expressed in terms of DiC_8-stimulated radioactivity, 72% was in DiC_8-PC whereas long-chain PC accounted for only 29%.

Similar results were obtained when long-chain PC and DiC_8-PC were separated by a published procedure (Liscovitch *et al.*, 1987) using a reversed-phase C18 TLC system (as described in section 2.21).

Fig. 41. Incorporation of DiC_8 into Phosphatidyl[*methyl*-^{3}H]choline. PKC - downregulated cells were prelabeled with 2 μCi/ml of [*methyl*-^{3}H]choline chloride for 1 h after which the cells were chased in the absence (open symbols) or presence (solid symbols) of DiC_8. 15 min later the cells were harvested, lipids extracted and long-chain PC separated from DiC_8-PC as described in section 2.21. The radioactivity in the long-chain PC bands (panel A) and in DiC_8-PC (panel B) was determined. The mass of PC and PE was also determined as shown in panel C.

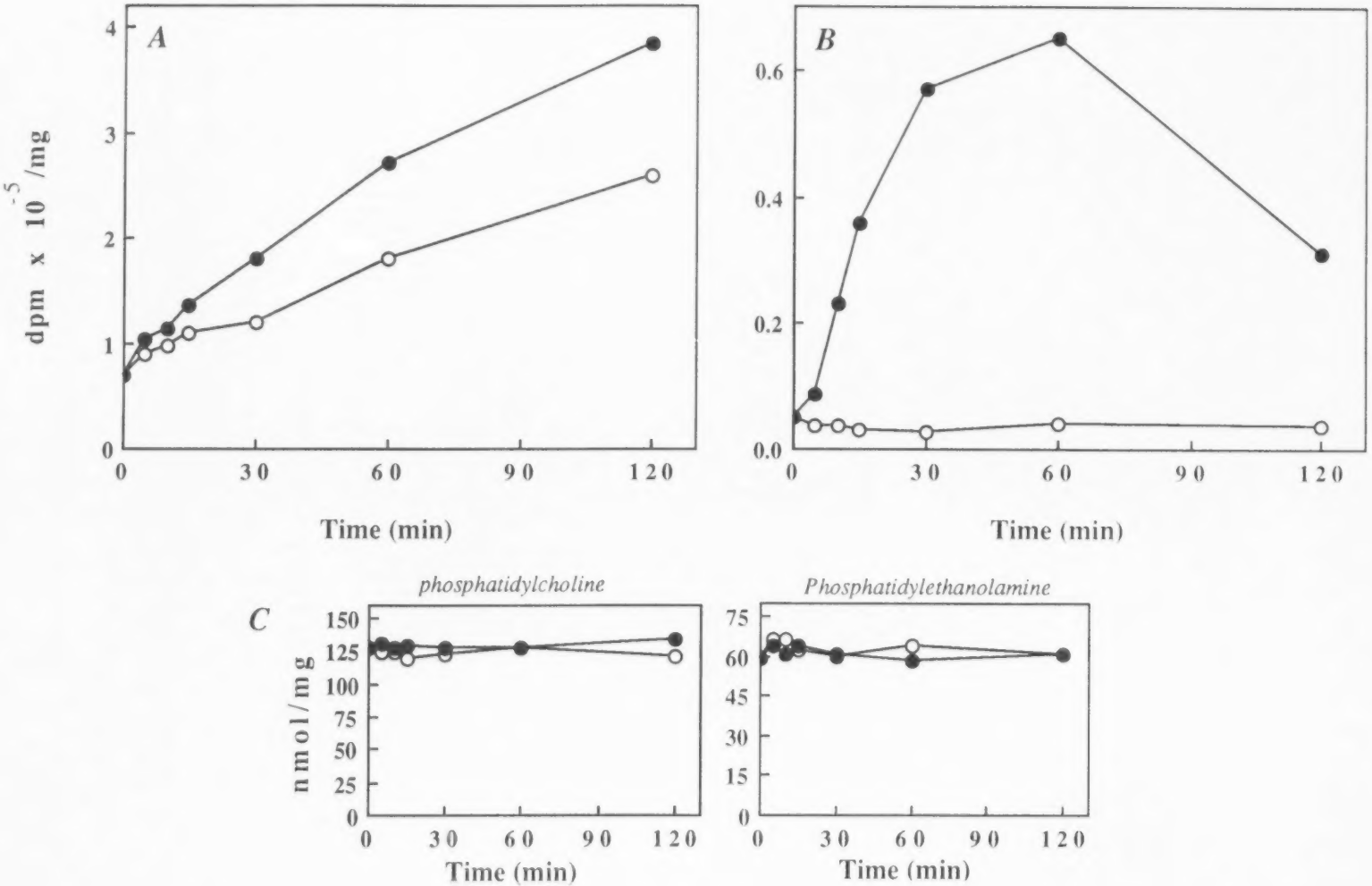

A
dpm x 10 -5 /mg
0
1
2
3
4
0
30
60
90
120
Time (min)
B
0.0
0.2
0.4
0.6
0
30
60
90
120
Time (min)
C
phosphatidylcholine
nmol/mg
0
25
50
75
100
125
150
0
30
60
90
120
Time (min)
Phosphatidylethanolamine
0
15
30
45
60
75
0
30
60
90
120
Time (min)

Table 3. Incorporation of DiC_8 into PC. PKC-downregulated cells were prelabeled for 1 h with 2 μCi/ml [^{3}H]choline. The labeled medium was replaced with medium ± 0.5 mM DiC_8 for 15 min amd the cells harvested. Lipids were extracted and separated by TLC in chloroform : methanol : acetic acid : water (70 : 30 : 12 : 4 : 2). The radioactivity in long-chain PC (R_F=0.25) and DiC_8-PC (R_F=0.17) was determined by scraping the silica followed by liquid scintillation counting.

% of total[a]			% of stimulated[b] (in DiC_8-treated cells)	
long-chain PC		DiC_8-PC		
control cells	DiC_8-treated cells	(in DiC_8-treated cells)	long-chain PC	DiC_8-PC
98 ± 1 (n=4)	60 ± 3 (n=5)	31 ± 5 (n=5)	29 ± 2 (n=3)	72 ± 3 (n=4)

a represents the % of radioactivity in the total lipid fraction

b represents the % of increased radioactivity in the total lipid fraction of DiC_8-treated cells (the control values were subtracted from DiC_8 values to give the increase in radioactivity due to DiC_8-treatment. The values represented are expressed as a % of this increase).

(b) The formation of DiC_8-PC as seen by 1,2-*sn*-dioctanoyl[2-^{3}H]glycerol labeling - PKC-downregulated cells were incubated with medium containing 2 μCi 1,2-*sn*-dioctanoyl[2-^{3}H]glycerol and 0.5 mM DiC_8. Cells were harvested after various times and lipids extracted. Lipids were separated by TLC and the radiolabel in PC and DiC_8 determined. As seen in Fig. 42, the level of radiolabeled DiC_8 decreased with a concomitant increase in labeled PC. After 15 min approximately 12% of the radioactivity in the total lipid fraction was found to be associated with DiC_8-PC, whereas 70% remained associated with DiC_8.

(c) Formation of DiC_8-PC as confirmed by GLC analysis - Gas liquid chromatography was used to confirm the incorporation of DiC_8 into PC by demonstrating the presence of 8:0 in DiC_8-PC. PKC-downregulated HeLa cells were incubated in the presence or absence of 0.5 mM DiC_8. After 15 min the cells were harvested, lipids extracted and DiC_8-PC separated from long-chain PC by TLC. Fig. 43 shows the retention times of a mixture of butyl fatty ester standards prepared by transesterification of a fatty acid mixture with BF_3-butanol (10%, w/w). The butyl 8:0 eluted with a retention time of 17.63 min. The presence of 8:0 in DiC_8-PC obtained from cells is shown in Fig. 44. The portion of the silica gel scraping corresponding to DiC_8-PC was scraped from the TLC plate used for separating the two forms of PC and subjected to transbutylation and subsequently to GLC analysis. A peak eluting with a retention time of 17.68 min was seen when samples from DiC_8-treated cells were analysed (panel B). This peak was absent from samples obtained from untreated control cells (panel A). To confirm further the identity of this peak, a sample of the butyl esters prepared from the DiC_8-treated cells was coinjected with a butyl 8:0 standard prepared from DiC_8 (Fig. 45). A single peak with a retention time of 17.73 was observed (Fig. 45, panel B). Panel A shows the

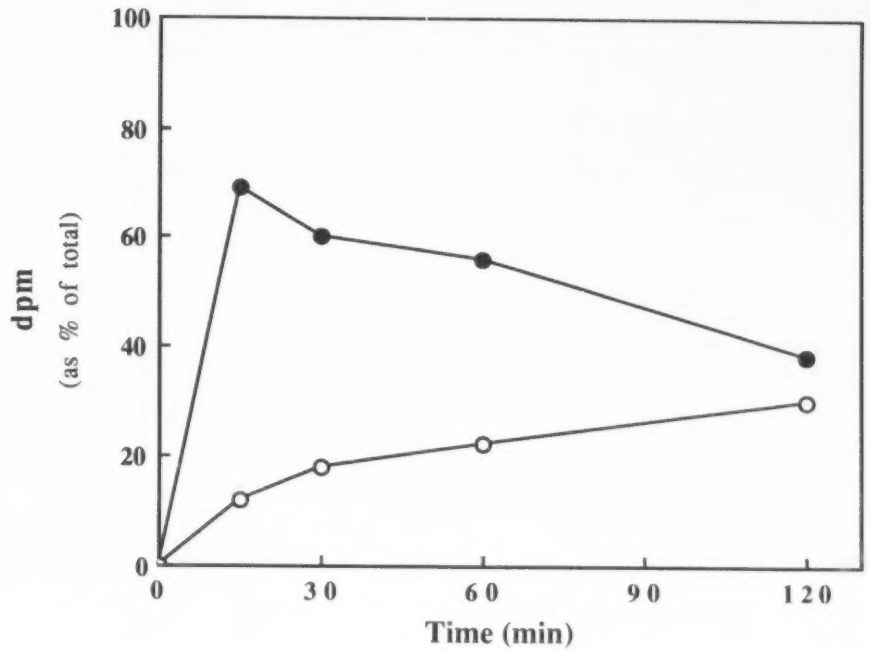


Fig. 42. Incorporation of 1,2-*sn*-dioctanoyl[2-^{3}H]glycerol into PC. PKC-downregulated HeLa cells in 35 mm dishes were incubated in medium containing 2 µCi 1,2-*sn*-dioctanoyl[2-^{3}H]glycerol. Cells were harvested at various times, lipids extracted and PC separated from DiC_8 by TLC. Phospholipids were separated by developing the TLC plate half way in chloroform : methanol : acetic acid : formic acid : water (70 : 30 : 12 : 4 : 2). Neutral lipids which migrated with the solvent front were separated by developing the same TLC plate to its full extent in diethyl ether : hexane : acetic acid (35 : 65 : 2). The bands on the silica gel corresponding to PC (open symbols) and DiC_8 (solid symbols) were subjected to liquid scintillation counting. The results are expressed as % of the radioactivity in the total lipid fraction.

Fig. 43. GLC Separation of Butyl Esters of Fatty Acids Prepared from a Mixture of Free Fatty Acid Standards. A mixture of fatty acids was butylated as described in section 2.22. 2 µl of the butyl-fatty esters were injected for GLC separation on a 6 feet x 0.125 inch stainless steel column packed with 10% DEGS on a 100/120 mesh Supelcoport™. The horizontal axis shows the retention time scale in min, and the vertical axis shows the oven temperature. The retention times of the various peaks are indicated on the chromatogram itself alongwith the fatty acid chain length. The peak at the beginning is due to the solvent.

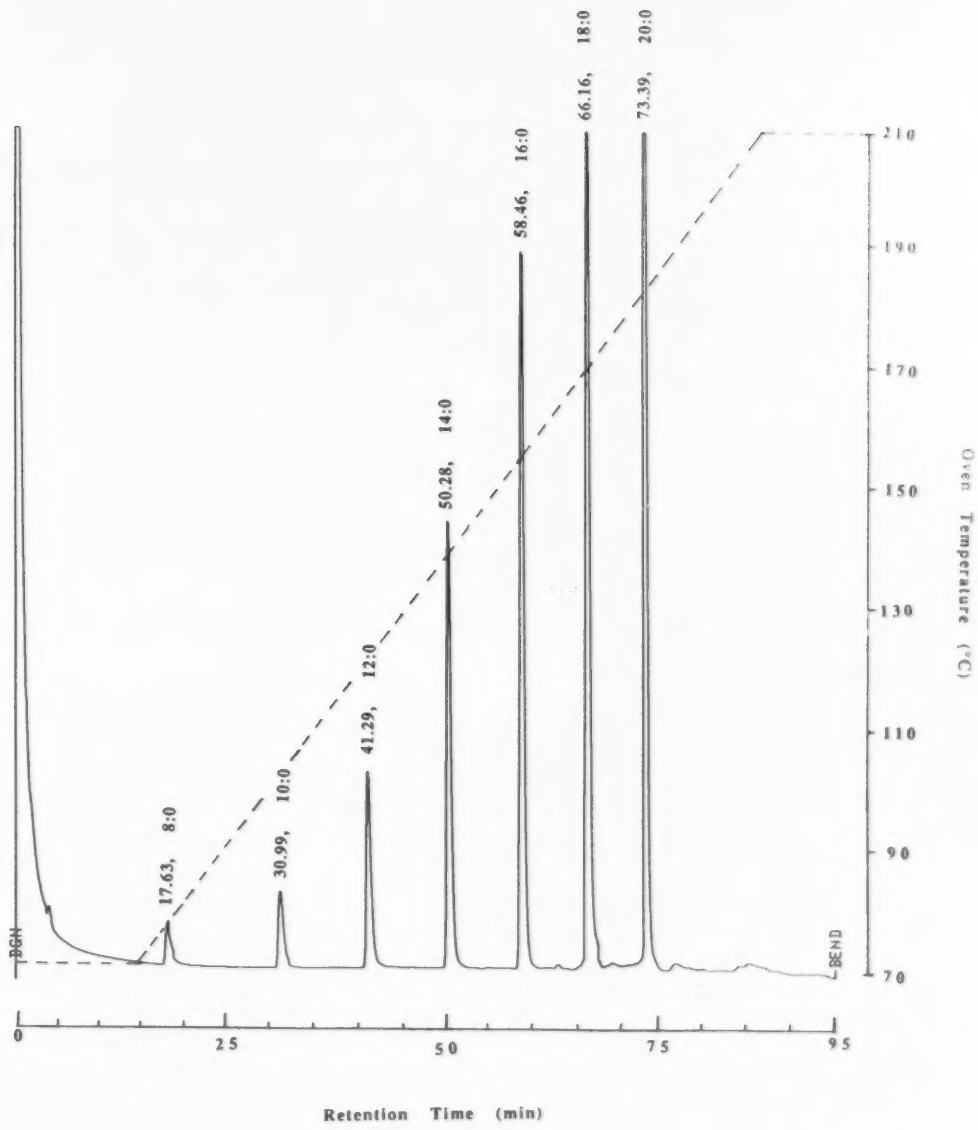

17.63, 8:0
30.99, 10:0
41.29, 12:0
50.28, 14:0
58.46, 16:0
66.16, 18:0
73.39, 20:0
BGN
BEND
0
25
50
75
95
70
90
110
130
150
170
190
210
Oven Temperature (°C)
Retention Time (min)

Fig. 44. GLC Separation of Butyl Esters of DiC_8-PC Showing the Presence of Octanoic Acid (8:0). 5, 100 mm, dishes of HeLa cells were down-regulated for PKC by incubation for 22 h in growth medium containing 1 μM TPA. The cells were subsequently incubated for 15 min in the absence (A) or presence (B) of 0.5 mM DiC_8 and harvested. Total lipids were extracted and DiC_8-PC separated from long-chain PC by TLC in chloroform : methanol : acetic acid : formic acid : water (70 : 30 : 12 : 4 : 2). Long-chain PC and DiC_8-PC exhibited R_F values of 0.11 and 0.25, respectively. The area corresponding to DiC_8-PC was scraped and subjected to trans-esterification in the presence of BF_3-butanol (10%, w/w) as described in section 2.22. The resulting hexane phase after extraction was evaporated under nitrogen to 20 μl, and 2 μl injected for GLC analysis. The peak at the beginning is due to the solvent. The horizontal axis shows the retention time scale and the individual peak retention times in min are indicated.

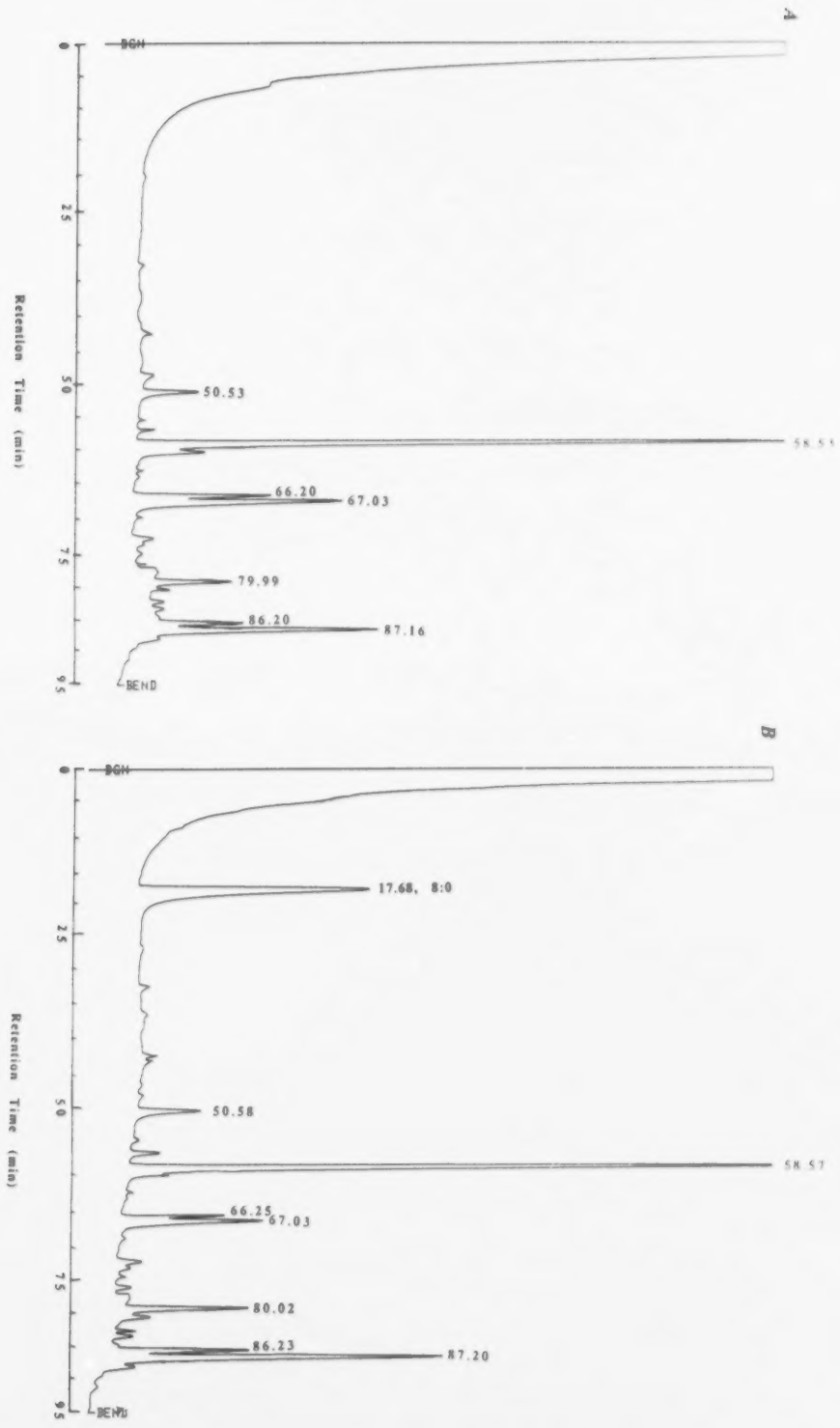
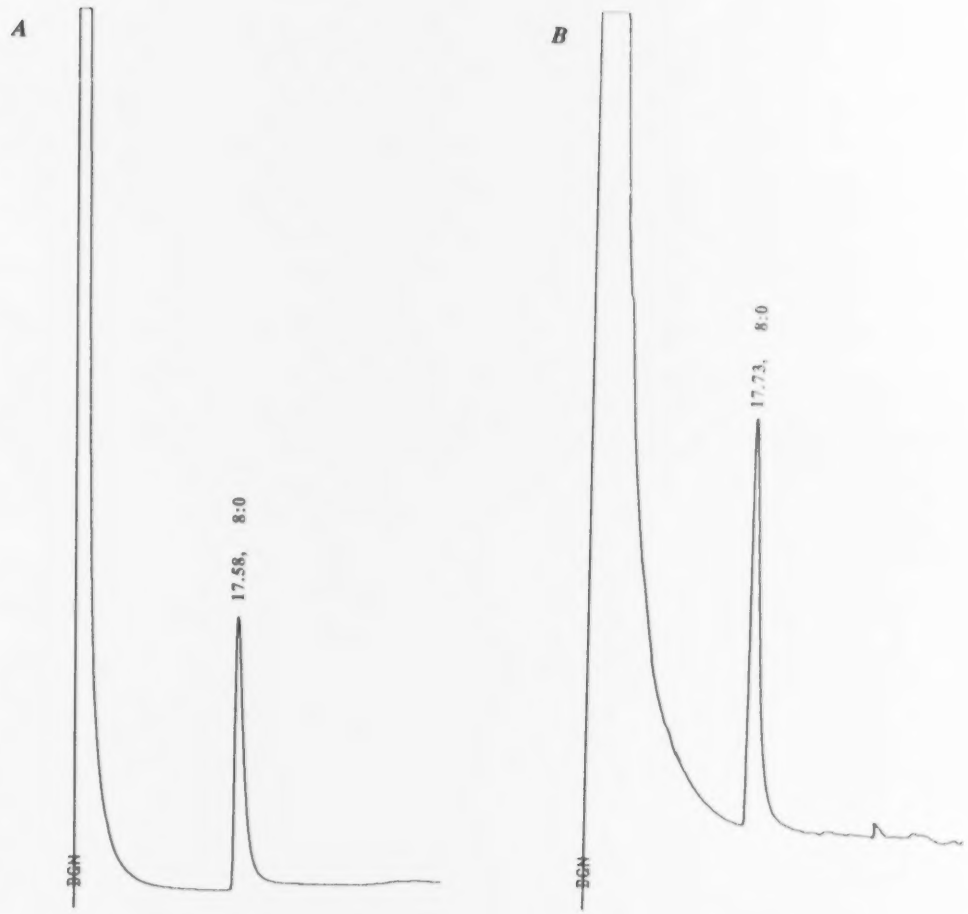

A
BGN
50.53
58.53
66.20
67.03
79.99
86.20
87.16
BEND
Retention Time (min)
0
25
50
75
95
B
BGN
17.68, 8:0
50.58
58.57
66.25
67.03
80.02
86.23
87.20
BEND
Retention Time (min)
0
25
50
75
95

Fig. 45. ***(A)*** **GLC Separation of the Butyl-Ester Standard of Octanoic Acid (8:0) Made from DiC_8.** 0.5 ml of the hexane phase after trans-butylation of DiC_8 was injected for GLC analysis. The butyl 8:0 peak eluted with a retention time of 17.58 min.

(B) **Co-injection of the Butyl-Ester Standard of Octanoic Acid Alongwith the Butyl Esters of Cellular DiC_8-PC.** 0.5 µl of butylated 8:0 standard was co-injected alongwith 2 µl of the butylated fatty acids of cellular DiC_8-PC obtained from PKC-downregulated cells treated with 0.5 mM DiC_8. The portion of the chromatogram with the butyl-8:0 peak (retention time = 17.73 min) is shown.


A
17.58, 8:0
BGN
B
17.73, 8:0
BGN

single peak obtained from an 8:0 standard prepared by transbutylation of DiC_8 (retention time = 17.58 min).

3.3.8 *Effect of Inhibitors of DG Metabolism on DG Formation*

As another approach to increasing intracellular DG levels, in addition to the DiC_8 experiments described in the previous sections, HeLa cells were treated with inhibitors of DG metabolism. Two inhibitors were used; (i) an inhibitor of platelet DG lipase (Sutherland and Amin, 1982) known as U-57908 (formerly RHC 80267, from the Upjohn Company) which has been shown to increase DG levels in gonadotropes by inhibiting DG hydrolysis (Chang *et al.*, 1988), and (ii) a DG kinase inhibitor (R-59022 purchased from Boerhinger Mannhiem) which has also been shown to enhance DG levels in thrombin-stimulated platelets by inhibiting the conversion of DG to PA (de Chaffoy de Courcelles *et al.*, 1985; Nunn and Watson, 1987). The chemical structure of both inhibitors are shown in Appendix C.

Pulse-chase experiments using these two inhibitors were performed in HeLa cells that had been prelabeled for 24 h with [2-^{3}H]glycerol. The labeled cells were incubated in medium ± inhibitors for 2 h and harvested. Lipids were extracted and neutral lipids separated by TLC. The amounts of radioactivity in DG were determined. An additional set of cells was treated with TPA as a positive control. The results in Fig. 46 show that TPA, as in section 3.2.1, caused increased formation of [^{3}H]DG (2.5 x 10^4 dpm/dish, lane 2) over that in control cells (1.5 x 10^4 dpm/dish, lane 1). However, the DG lipase (lane 3) but not the DG kinase inhibitor (lane 4) showed only a slight increase in [^{3}H]DG. Both inhibitors together increased [^{3}H]DG to the same level as DG lipase inhibitor alone (1.9 x 10^4 dpm/dish, lane 5), but this was still lower than the increase caused by TPA alone. As seen in lanes 6, 7 and 8 inclusion of the

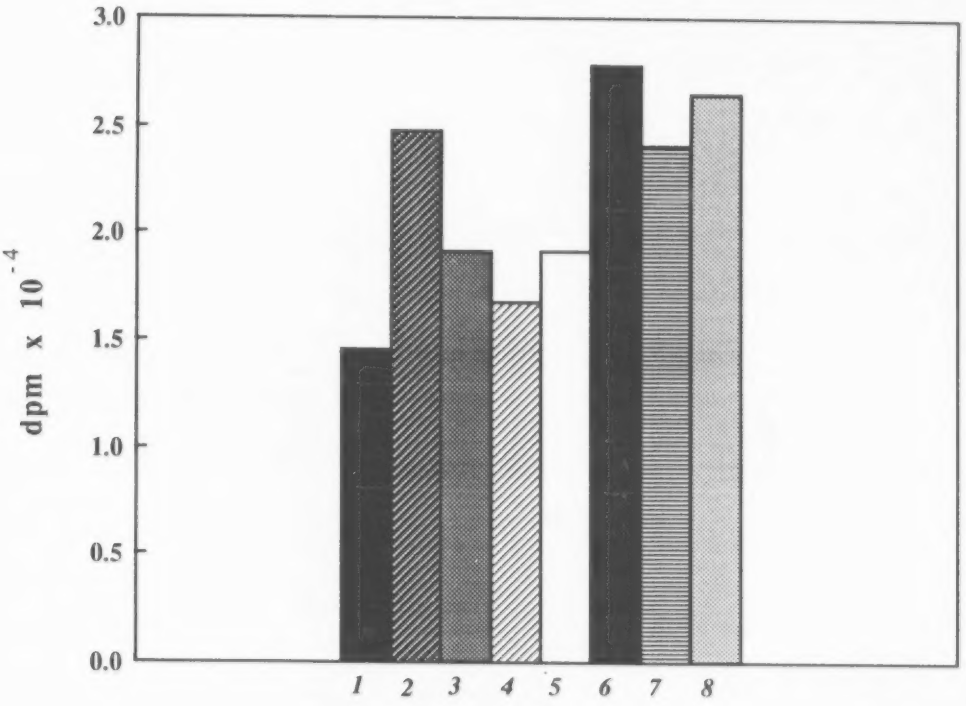


Fig. 46. Effect of U-57908 and R-59022 on the Accumulation of [^{3}H]DG. HeLa cells were prelabeled with 2 μCi/ml [2-^{3}H]glycerol for 24 h at which time the labeled medium was removed and the cells were incubated in the absence (Control, lane 1) or presence of 100 nM TPA (lane 2), 10 μM U-57908 (lane 3), 10 μM R-59022 (lane 4), U-57908 + R-59022 (lane 5), TPA + U-57908 (lane 6), TPA + R-59022 (lane 7) and TPA + U-57908 + R-59022 (lane 8). After 2 h of chase cells were harvested, lipids extracted and separated in diethyl ether : hexane : acetic acid (35 : 65 : 2). The radioactivity in the DG was determined and expressed as total dpm x 10^4/dish.

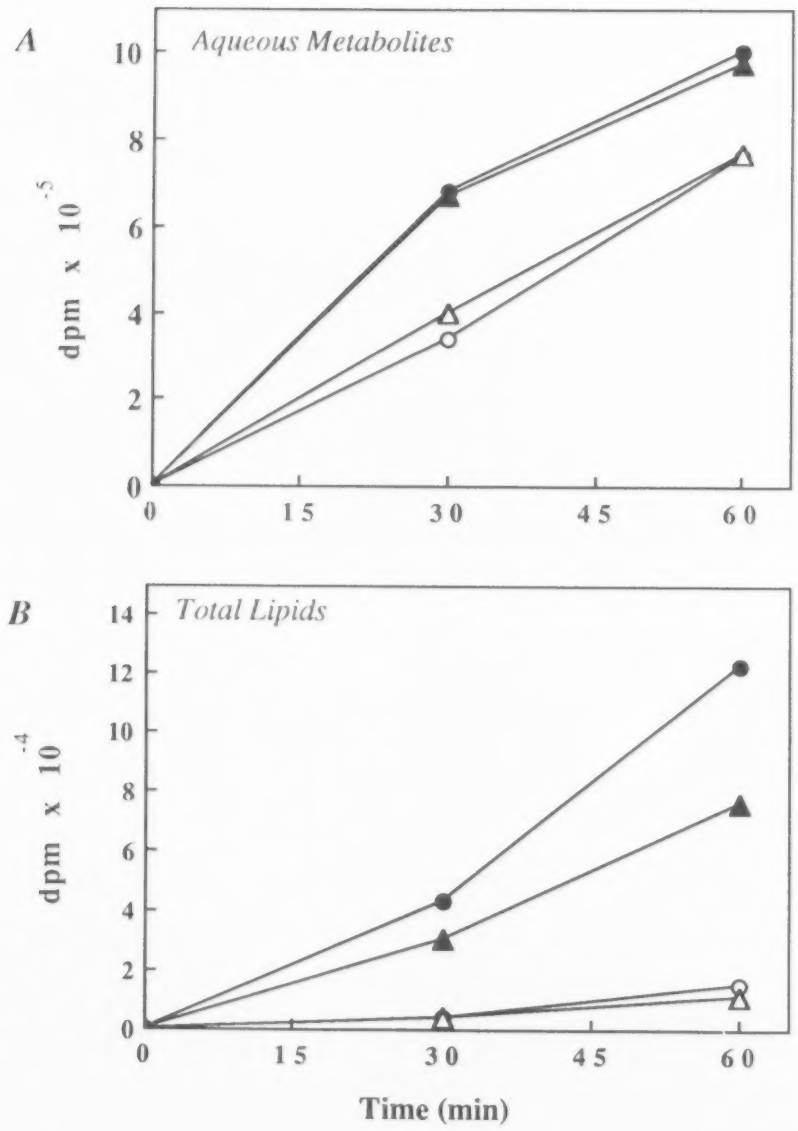
inhibitors alongwith TPA did not enhance the accumulation of [^{3}H]DG except for DG lipase which enhanced the TPA-mediated increase slightly to 2.8 x 10^4 dpm/dish. Since the effects obtained with the inhibitors were minor compared to those obtained with TPA, no further studies with the inhibitors were attempted.

3.3.9. *Effect of U-73122 On TPA-Stimulated PC Biosynthesis*

To further investigate the role of DG in CT translocation and PC biosynthesis experiments were performed with U-73122 (for chemical structure see Appendix C), a phospholipase C inhibitor (from the Upjohn Company, Kalamazoo, MI). Since there was some evidence that the DG formation due to TPA-treatment was possibly a phospholipase C - mediated hydrolysis of PC (Glatz *et al.*, 1987), an attempt was made to inhibit this formation of DG and, therefore, to delineate the role of DG.

Initially, continuous labeling experiments were performed in the absence or presence of U-73122 ± TPA. Cells were preincubated in the presence of U-73122 for 10 min before incubating in medium containing 2 μCi/ml [*methyl*-^{3}H]choline chloride and 5 μM U-73122 ± 100 mM TPA. Controls were preincubated in medium alone and then incubated in labeled medium ± 100 nM TPA. Cells were harvested after 30 and 60 min, lipids were extracted and the radioactivity in the aqueous choline metabolites and the total lipid fraction determined. The results are shown in Fig. 47. U-73122 did not appear to have any effect on the incorporation of label into the aqueous choline metabolites (panel A) or into the total lipid fraction (Panel B). However, the inclusion of U-73122 alongwith TPA appeared to decrease the TPA-stimulated incorporation of [^{3}H]choline-labeled precursors into PC. In agreement with

Fig. 47. Effect of U-73211 on [^{3}H]Choline Incorporation. HeLa cells were preincubated in the absence (circles) or presence (triangles) of 10 μM U-73122 for 10 min before incubating in the presence of medium containing 2 μCi/ml [*methyl*-^{3}H]choline chloride, and 100 nM TPA ± 10 μM U-73122. Cells were harvested at 30 min and 60 min, lipids extracted and the radioactivity in the choline-containing aqueous metabolites (panel A) and the total lipid fraction (panel B) determined. The open and solid symbols represent incubations in the absence or presence of TPA, respectively. Incubations in the absence or presence of U-73122 are represented by circles and triangles, respectively.


A
Aqueous Metabolites
dpm x 10^-5
10
8
6
4
2
0
0
15
30
45
60
B
Total Lipids
dpm x 10^-4
14
12
10
8
6
4
2
0
0
15
30
45
60
Time (min)

Kinzel *et al.* (1979) TPA increased the incorporation of [^{3}H]choline into HeLa cells.

Pulse-chase experiments were performed so that all the cellular choline metabolites were labeled equally before incubation of the cells with agents and to follow the progress of the labeled metabolites. The results are shown in Fig. 48. HeLa cells were prelabeled with 2 µCi/ml [*methyl*-^{3}H]choline chloride. After 1 h the labeled medium was replaced with unlabeled medium and the cells were preincubated for 10 min in the presence of 10 µM U-73122. Subsequently the preincubation medium was removed and the cells incubated in medium containing 10 µM U-73122 in the absence (lane 4) or presence (lane 5) of TPA. The various controls were preincubated for 10 min in the absence of U-73122, and the subsequent chase was also in the absence of the inhibitor (lanes 1, 2 and 3). Cells were harvested after 1 h, lipids extracted and the radioactivity in the total lipid fraction determined. As expected, the positive control incubated in the presence of TPA alone showed a stimulation of PC biosynthesis (lane 3) over that of the controls without agents (lanes 1 and 2). U-73122 had no effect on the TPA-stimulated incorporation of radiolabel into the total lipid fraction (lane 5), and was without effect when it was present alone (lane 4).

In light of the lack of effect of U-73122 on TPA-mediated stimulation of PC biosynthesis as seen in the above experiments, experiments involving this inhibitor were not pursued further.

At the time these experimants with U-73122 were done, Bleasdale *et al.* (1990) reported that the inhibitor did not affect TPA-stimulated DG production. This explained the lack of effect of U-73122 on TPA-stimulated PC metabolism. The specificity of this inhibitor towards PI-specific phospholipase C was also reported (Smith *et al.*, 1990), again supporting observations in the literature

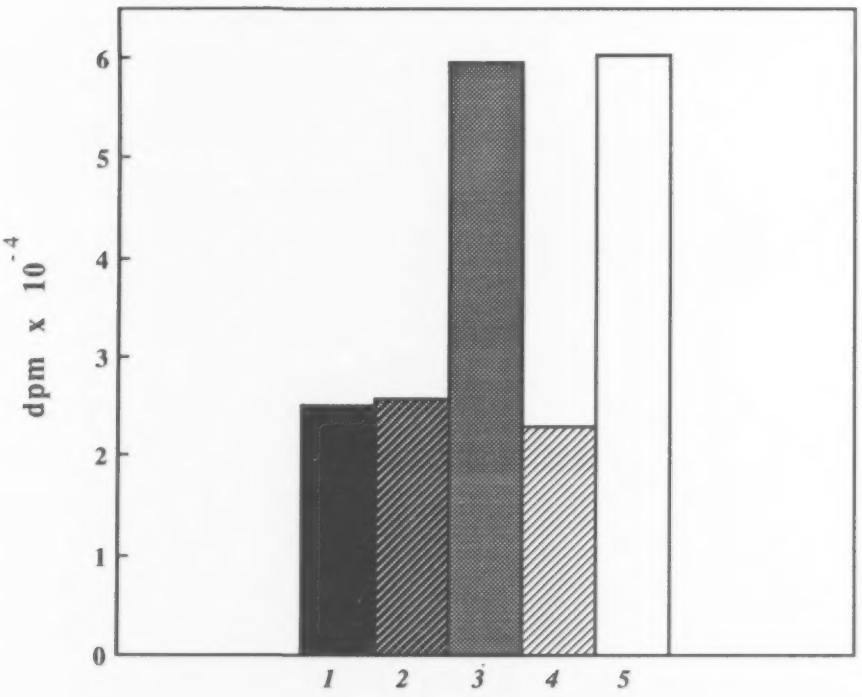


Fig. 48. Lack of Effect of U-73122 on TPA-Stimulated PC Biosynthesis. HeLa cells were prelabeled with 2 μCi/ml [*methyl*-^{3}H]choline chloride for 1 h at which time the labeled medium was removed and the cells preincubated for 10 min in the absence (lanes 1, 2, 3) or presence (lanes 4 and 5) of 10 μM U-73122. Subsequently, the cells were chased in medium alone (lane 1), medium containing 10 μM U-73122 (lane 2), 100 nM TPA (lane 3), U-73122 minus 100 nM TPA (lane 4) and U-73122 plus TPA (lane 5). After 1 h of chase cells were harvested, lipids extracted and the radioactivity in the total lipid fraction determined. Controls which were not treated with U-73211 are represented by lanes 1, 2, and 3. Lanes 4 and 5 represent cells that were preincubated with U-73211 followed by a chase in the absence or presence TPA, respectively.

that TPA-stimulated PC hydrolysis is due to an enzyme distinct from PI-specific phospholipase C.

3.4 ELIMINATION OF OTHER POSSIBLE MECHANISMS OF CT TRANSLOCATION DUE TO TPA

Several other mechanisms as possible mediators of CT translocation to membranes were investigated. A wide range of eukaryotic and viral proteins are known to undergo post-translational covalent modifications (Cross, 1987; Merrill, 1989; Glomset *et al.*, 1990). Possible covalent modifications of CT such as reversible phosphorylation and covalent attachment of lipid moieties were investigated as described below. Aggregation of CT as a non-covalent modification was also investigated as was the effect of choline deprivation on the subcellular distribution of CT.

3.4.1 *Reversible phosphorylation*

Several lines of evidence indicated that the subcellular distribution of CT may be regulated by a reversible phosphorylation mechanism. The studies of Pelech *et al.* (1981) have shown that incubation of rat hepatocytes with stable analogues of cAMP inhibited PC biosynthesis and was shown to be accompanied by a decrease in microsomal CT activity accompanied by a redistribution of CT to the cytosol. *In vitro* evidence for a reversible phophorylation mechanism was provided by Sanghera and Vance (1989) in which purified rat liver CT was shown to translocate to membranes upon dephosphorylation with alkaline phosphatase. In the same study it was demonstrated that pure rat liver CT could be phosphorylated with cAMP-dependent protein kinase. More recently, okadaic acid, a specific inhibitor of

protein phosphatases 1 and 2A has been used in this laboratory to increase intracellular phosphorylation states (Hatch, G.M. and Vance, D.E., unpublished observations) and to probe the effect of increased phosphorylation on the subcellular distribution of CT and PC biosynthesis. Treatment of hepatocytes with okadaic acid inhibited PC biosynthesis by 29%. This was accompanied by a 37% decrease in membrane-associated CT activity and its redistribution to the cytosol (Hatch *et al.*, 1991). These studies, however, only provided indirect evidence for the role of a reversible phosphorylation mechanism for CT as it was not possible to demonstrate whether the changes in the subcellular distribution of CT activity were accompanied by an altered phosphorylation state of CT due to the lack of a precipitating antibody to CT. Direct studies on the phosphorylation state of CT only became possible with the successful production of an antibody that precipitated CT. Rabbit antibodies were generated against a synthetic peptide sequence (amino acid residues 164 to 174 of rat liver CT) as described in section 2.16.

(a) Immunoprecipitation of CT from ^{32}P-labeled HeLa cells - Since HeLa cells were treated with TPA which activates PKC, it was possible that a phosphorylation event directly influenced CT translocation. A direct phosphorylation of CT by protein kinase C or another kinase appeared unlikely as this was contrary to the hypothesis that dephosphorylation of CT causes CT to associate with membranes. Moreover, TPA caused CT to translocate to membranes which would *not* agree with a phosphorylation of CT mediating translocation as phosphorylation would make CT more hydrophillic and less likely to associate with membranes. However, it *was* possible that PKC activated a phosphatase that would dephosphorylate CT and therefore, cause its translocation to membranes in TPA-treated cells. In a recent report Watkins

and Kent (1990) showed for the first time by immunoprecipitation methods that cytosolic CT from HeLa cells is phosphorylated.

Therefore, experiments were performed to investigate the role of phosphorylation, if any, in CT translocation in HeLa cells activated for PKC. CT was immunoprecipitated from ^{32}P-labeled cells treated in the absence or presence of TPA (Fig. 49). The immunoprecipitated proteins were separated by SDS-PAGE and the gel was exposed for autoradiography. TPA treatment did not result in any significant difference in the phosphorylation state of cytosolic CT (lanes *1* and *2*). Immunoprecipitated particulate CT from TPA-treated cells (lane *6*) showed a 50% increase in intensity (as measured by densitometric scanning) over that from control cells (lane *5*). Since samples were immunoprecipitated from equal amounts of particulate protein this increase is most likely due to the increased mass of particulate CT elicited by TPA. A similar result (Fig. 50) was obtained when the immunoprecipitations were repeated in the presence of 25 mM sodium fluoride, 2 mM EDTA and 1 mM EGTA, conditions that inhibit phosphatase activity.

Therefore, these results do not indicate an obvious role for a reversible phosphorylation mechanism in TPA-elicited CT translocation, although the enzyme is highly phosphorylated in the presence and absence of TPA.

Furthermore, there is no evidence that purified rat liver CT is phosphorylated by protein kinase C, again indicating the lack of a regulatory mechanism by phosphorylation of CT via PKC (Jamil, H. and Vance, D.E., unpublished observations).

(b) <u>The lack of an effect of okadaic acid on TPA-stimulated PC biosynthesis</u> - As another approach to studying the effects of increased phosphorylation on CT okadaic acid was used. Okadaic acid is a polyether fatty acid which was first isolated from the marine sponges *Halichondria okadii* and

Fig. 49. Immunoprecipitation of CT from ^{32}P-Labeled HeLa Cells.

Confluent HeLa cells were labeled with ^{32}Pi for 2 h and incubated in the absence or presence of TPA for a further 1 h. The cells were digitonin-permeabilized and CT was immunoprecipitated from released cytosol and particulate fractions with a rabbit polyclonal antibody to CT. 0.5% BSA was included in the incubations to eliminate non-specific binding. The immunoprecipitated CT samples were separated on SDS-PAGE which was subjected to autoradiography (exposure at -70°C for 5 days in a film cassette with intensifier screens). Immunoprecipitation of cytosolic CT is shown in lanes *1* and *2*, non-specific immunoprecipitation of cytosolic CT with unadsorbed IgG in lanes *3* and *4* and immunoprecipitation of particulate CT in lanes *5* and *6*. Lanes *1*, *3* and *5* show samples from untreated control cells, lanes *2*, *4* and *6* show samples from TPA-treated cells.

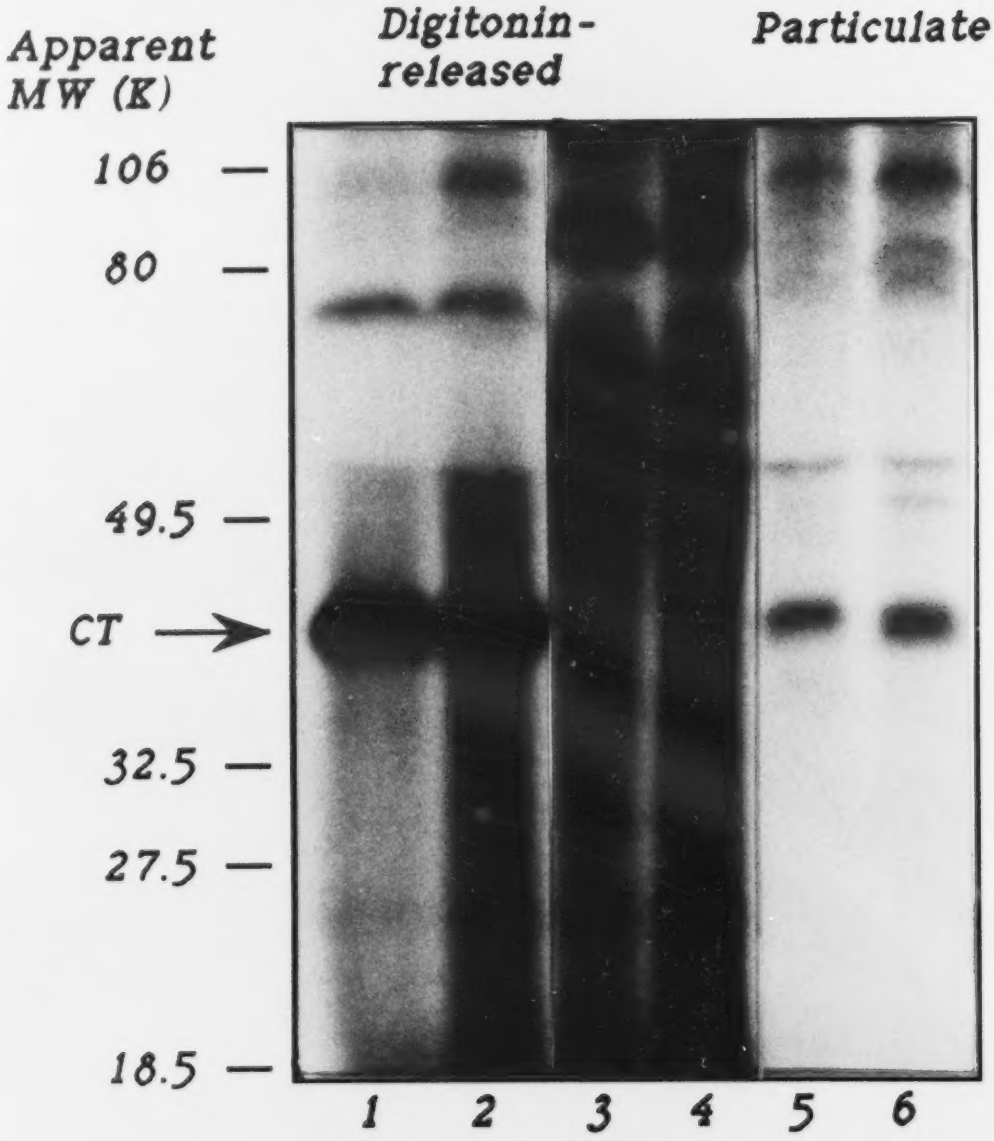
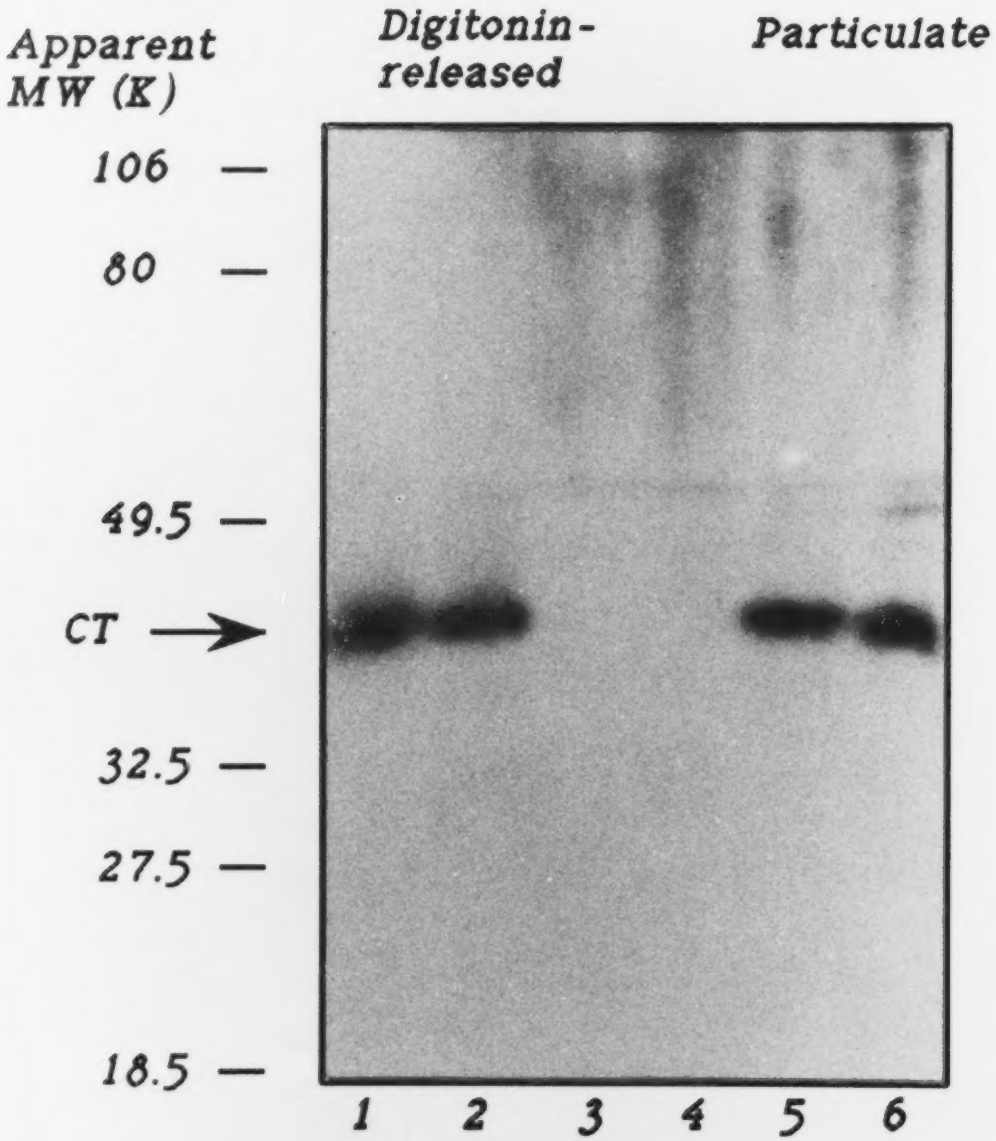

Apparent MW (K)
Digitonin-released
Particulate
106
80
49.5
CT
32.5
27.5
18.5
1
2
3
4
5
6

Fig. 50. Immunoprecipitation of CT From ^{32}P-Labeled HeLa Cells in the Presence of Phosphatase Inhibitors. Confluent HeLa cells were labeled with ^{32}Pi for 2 h and incubated in the absence or presence of TPA for a further 1 h. The cells were digitonin-permeabilized and CT was immunoprecipitated from 200 µl released cytosol and total particulate fractions with a rabbit polyclonal antibody to CT in the presence of 25 mM NaF, 2 mM EDTA and 1 mM EGTA. 1% BSA was included in the incubations to eliminate non-specific binding. The immunoprecipitated CT samples were separated on SDS-PAGE which was subjected to autoradiography (exposure at -70°C for 2 days in a film cassette with intensifier screens). Immunoprecipitation of cytosolic CT is shown in lanes *1* and *2*, non-specific immunoprecipitation of cytosolic CT in lanes *3* and *4* and immunoprecipitation of particulate CT in lanes *5* and *6*. Lanes *1*, *3* and *5* show samples from untreated control cells, lanes *2*, *4* and *6* show samples from TPA-treated cells.


Apparent MW (K)
Digitonin-released
Particulate
106
80
49.5
CT
32.5
27.5
18.5
1
2
3
4
5
6

Halichondria melanodocia by Tachibana *et al.* (1981). It is a potent and specific inhibitor of protein phosphatases 1 (PP1) and 2A (PP2A), two of the four major cytosolic phosphatases in mammalian cells (for a review see Cohen *et al.*, 1990). It was proposed that okadaic acid might reverse TPA-stimulated PC biosynthesis in HeLa cells by inhibiting the phosphatases that might have been responsible for any dephosphorylation of CT if such a mechanism were operative. As a preliminary study to investigate this pulse-chase experiments were conducted with HeLa cells ± agents.

Addition of okadaic acid to cultured hepatocytes resulted in the cells assuming a rounded shape and not adhering to the tissue culture dishes. Therefore, experiments in this laboratory involving okadaic acid were performed on hepatocyte suspensions, rather than on adherent cultured hepatocytes (Hatch *et al.*, 1991). The effect of various concentrations of okadaic acid on HeLa cell adherence was assessed by light microscopy. For upto 50 min 10 nM, 100 nM and 1μM okadaic acid did not cause cells to 'lift off' from the tissue culture dishes. After 50 min cells in 1 μM okadaic acid showed signs of rounding up but still remained adhered to the dishes. Cells in 100 nM okadaic acid showed signs of rounding up only after 5 h whereas cells in 10 nM okadaic acid appeared to be morphologically unaffected throughout this time period. Cells were viable at all times as seen by trypan blue exclusion.

HeLa cells were prelabeled with 2 μCi/ml [*methyl*-^{3}H]choline chloride for 1 h followed by a chase of 40 min ± agents (Fig. 51). Cells were harvested, lipids extracted and the radiolabel in the total lipid fraction determined. Lanes 1 and 2 are controls which were chased with medium alone and medium with 0.25% DMSO (the final concentration of DMSO when TPA and okadaic acid were present together), respectively. The results show that okadaic acid was without effect (lane 4) and did not reverse TPA-stimulated PC biosynthesis

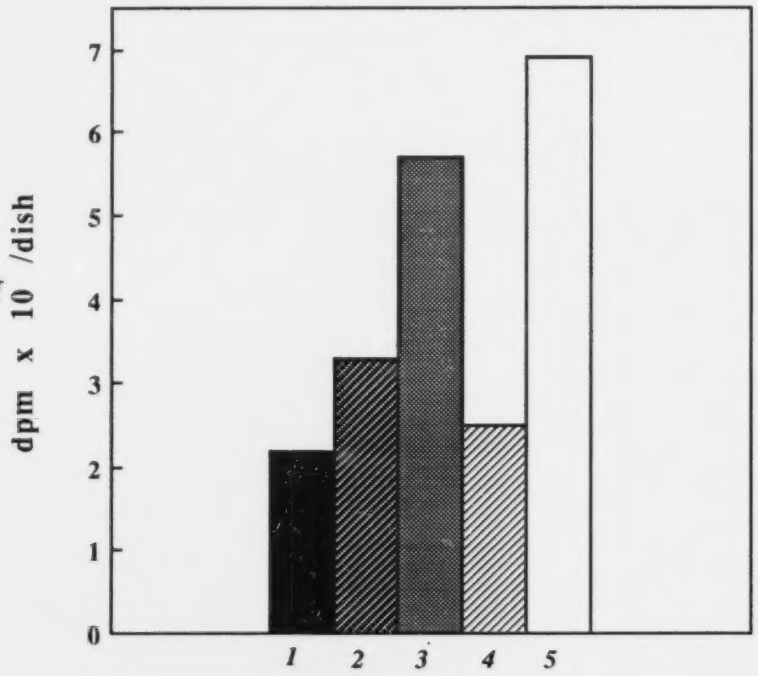


Fig. 51. Effect of Okadaic Acid on TPA-Stimulated PC Biosynthesis. HeLa cells were prelabeled with 2 μCi/ml [*methyl*-^{3}H]choline chloride for 1 h at which time the labeled medium was removed and the cells rinsed thrice. The cells were chased in the absence or presence of 100 nM TPA ± 1 μM okadaic acid. After 40 min the cells were harvested, lipids extracted and the radioactivity in the total lipid fraction determined. Lanes 1 and 2 represent controls which were incubated without agents in the absence or presence of 0.25% DMSO, respectively (0.25% DMSO was the highest concentration of DMSO present when TPA and okadaic acid were present together as in lane 5). Lane 3 represents cells that were chased in the presence of 100 nM TPA alone, lane 4 represents cells that were chased in the presence of okadaic acid alone and lane 5 represents cells that were incubated in the presence of both TPA and okadaic acid.

(lane 5). On the contrary, okadaic acid was seen to slightly increase the TPA-effect from 5.7 x 10^4 dpm (chase medium with TPA alone, lane 3) to 6.9 x 10^4 dpm (chase medium with both TPA and okadaic acid, lane 5). This increase can, however, be attributed to the presence of 0.25% DMSO which on its own slightly increased incorporation of [^{3}H]choline into the total lipid fraction from 2.2 x 10^4 dpm (control, lane 1) to 3.3 x 10^4 dpm (lane 2).

Since these preliminary results showed that okadaic acid had no effect on TPA-stimulated PC biosynthesis, no further experiments with okadaic acid were conducted.

3.4.2 *Covalent Lipid Modification*

The possibility that TPA-elicited CT translocation to membranes was due to covalent lipid modifications was addressed. The commonly known lipid modifications of proteins are covalent linkage of phosphatidylinositol (for a review see Cross, 1987), myristoylation, palmitoylation and covalent attachment of precursors of cholesterol biosynthesis such as farnesyl, isoprenyl or geranyl groups. The amino acid requirement or the consensus sequences for these modifications to occur are fairly well characterized in the literature. The cloning of rat liver CT by Cornell *et al.* (1990) showed that the deduced amino acid sequence did not possess the structural features generally required for covalent lipid modifications to occur and, therefore, the possibility of CT undergoing these modifications in TPA-treated HeLa cells was not rigorously pursued. Moreover, an immunoprecipitating antibody which was required for the facilitation of most of these studies became available only recently.

However, a preliminary study of one of these modifications was possible, albeit in an indirect fashion. Modification of protein by long chain prenyl

groups requires the biosynthesis of mevalonic acid. Inhibition of isoprenoid biosynthesis by the use of an inhibitor of HMGCoA reductase such as mevinolin can block the maturation of $p21^{ras}$ in HeLa cells (Leonard *et al.*, 1990). $p21^{ras}$ requires isoprenylation for maturation, and since the inhibition of isoprenoid biosynthesis has been well characterized in these studies from the laboratory of Dr. Michael Sinensky, experiments were performed in which the effect of inhibition of cholesterol precursors on TPA-stimulated PC biosynthesis and CT translocation was investigated.

Effect of isoprenoid biosynthesis on PC biosynthesis and CT translocation - HeLa cells in growth medium were treated with 20 μM lovastatin, a potent inhibitor of HMGCoA reductase, for 24 h. At the end of this time period cells were prelabeled with 2 μCi/ml [*methyl*-^{3}H]choline chloride for 1 h, after which the labeled medium was removed and the label chased ± TPA. Cells were harvested and the radioactivity in the total lipid fraction was determined. 10 μM lovastatin was present in all the incubations. TPA-stimulated incorporation of [^{3}H]choline into PC (from 1.5 x 10^4 dpm in controls to 3.8 x 10^4 dpm in TPA-treated cells) was not blocked in cells that had been starved of mevalonate by lovastatin treatment.

Parallel experiments were conducted to examine the effect of mevalonate-deprivation on TPA-elicited CT translocation. Cells which had been treated with 20 μM lovastatin for 24 h were incubated ± 100 nM TPA for 1 h. Subsequently the cells were digitonin-permeabilized and CT activity in the particulated fraction determined. TPA caused CT to translocate to membranes from 0.086 nmol/min/dish in control cells to 0.216 nmol/min/dish which is similar to the effect observed in cells that are not impaired for mevalonate biosynthesis.

These preliminary results indicated a lack of involvement of prenylation or covalent modification of CT by precursors of cholesterol biosynthesis in TPA-stimulated CT translocation and PC biosynthesis. It also appears from these results that cholesterol metabolism is unconnected with PC biosynthesis since impairment of mevalonate did not appear to affect the levels of PC labeling or of membrane CT compared to cells that were not treated with lovastatin.

3.4.3 *Lack of Aggregation of Cytosolic CT in TPA-Treated HeLa Cells*

CT has been shown by Weinhold *et al.* (1989) to exist in two forms in the cytosol of rat lung, HepG2 cells, A549 cells and alveolar Type II cells; a low molecular weight dimeric form of MW 45,000, the so-called L-form which is dependent on lipids for activation, and an aggregated H-form which is a lipoprotein complexed with lipids and is independent of added lipids for activity. Lipids such as phosphatidylinositol, oleic acid and phosphatidylglycerol caused the L-form to aggregate to the H-form *in vitro* and a change in the relative distribution of these two forms of CT has been suggested to be important in the regulation of PC biosynthesis.

Therefore, the existence of the L and H forms of CT in HeLa cells was investigated before and after treatment of the cells with TPA. HeLa cells were incubated in the absence (control) or presence of 100 nM TPA for 1 h. The cells were Dounce homogenized and the resulting homogenate was centrifuged at 99,000 rpm using a TL100.2 rotor in a Beckman TL100 ultracentrifuge. The supernatant was saved as cytosol and equivalent amounts of protein (0.65 mg) were layered onto 8 to 40% (v/v) linear glycerol gradients. Centrifugation was performed in a SW 40 rotor at 40,000 rpm for 14 h at 4°C in an L8-M ultracentrifuge. Fig. 52 shows that both the cytosol of untreated controls

(panel A) and TPA-treated cells (panel B) exhibited only the L form of CT. The activities in the various fractions after glycerol gradient centrifugation could account for the total activities loaded onto the gradients. Therefore, all the activities loaded were accounted for by the L form of CT. Rat liver and rat lung cytosol were also subjected to glycerol gradient centrifugation. As reported by Weinhold *et al.* (1989), rat liver cytosol exhibited only the L form (Fig. 53, panel A), whereas rat lung cytosol had both the L and H forms of CT (Fig. 53, panel B).

These results indicate that CT is present as the L form in HeLa cell cytosol and does not aggregate into the H form upon treatment of the cells with TPA.

3.4.4. *Lack of Evidence for Subcellular Redistribution of CT Due to Changes in Intracellular PC Levels*

Recent evidence from this laboratory suggested that intracellular levels of PC could influence the subcellular distribution of CT in hepatocytes. Yao *et al.* (1990) showed that there was 2-fold greater microsomal CT activity in choline-deficient rat livers compared to that in livers from rats fed a normal diet. When hepatocytes prepared from choline deficient rats were cultured in choline-supplemented medium a decrease in microsomal CT was seen with a concomitant increase in cytosolic CT activity. In another study by Jamil *et al.* (1990) it was demonstrated that choline-deficient hepatocytes had decreased PC levels compared to hepatocytes from normal rats, and that when PC levels were raised by various means microsomal CT activity redistributed to the cytosol. In the same study a highly significant inverse relationship was established between microsomal PC levels and the amount of microsomal CT. Therefore, the intracellular content of PC provided a level of control over the subcellular

Fig. 52. Analysis of Forms of CT in HeLa Cell Cytosol by Glycerol Gradient Centrifugation. Cytosolic samples were obtained form HeLa cells incubated for 1 h in the (*A*) absence or (*B*) presence of 100 nM TPA. 0.65 mg of protein was layered onto a 12 ml, 8 - 40% (v/v) linear glycerol gradient and centrifuged at 40,000 rpm for 14 h at 4°C. 0.5 ml aliquots were removed starting from the top of the tube and assayed for CT activity.

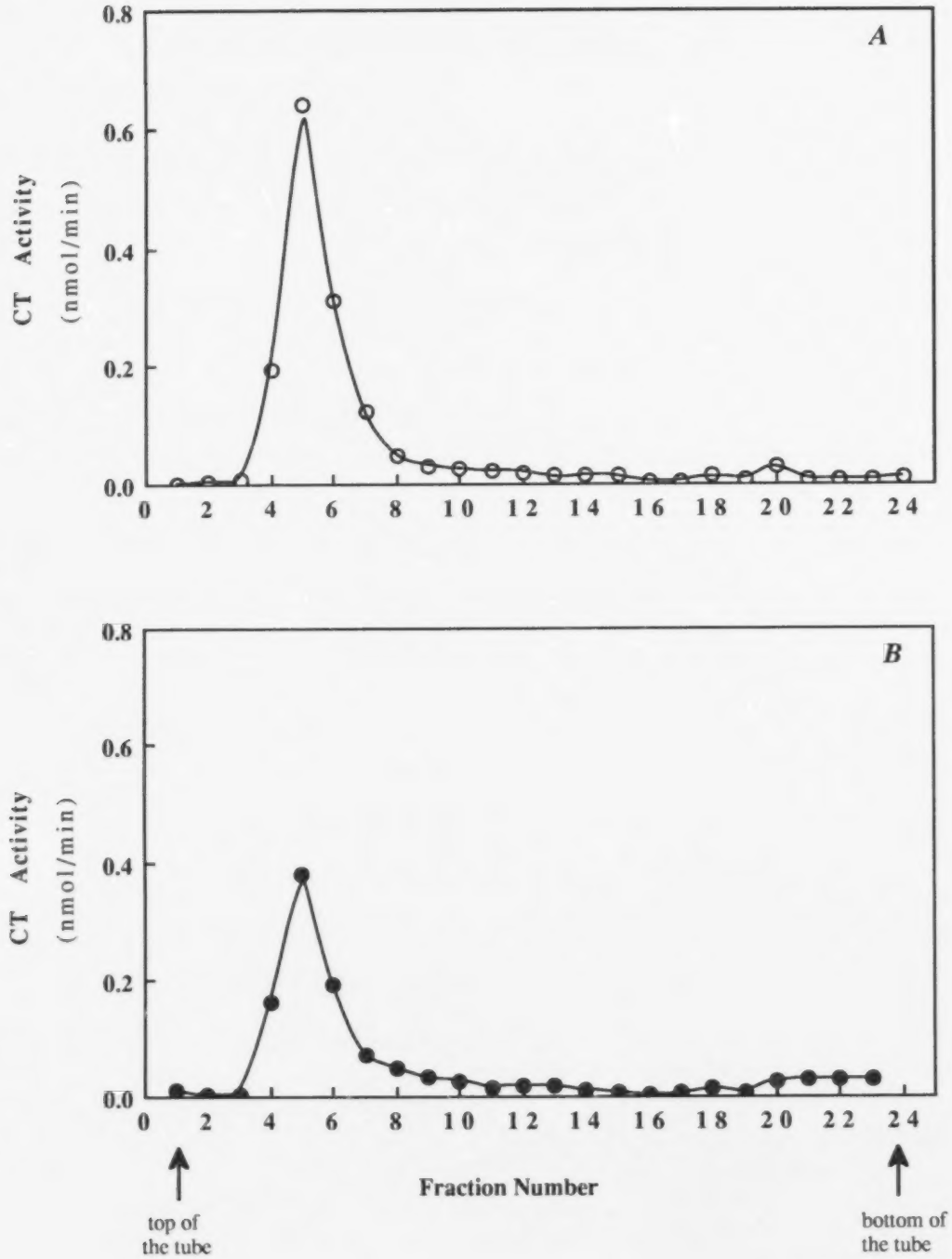
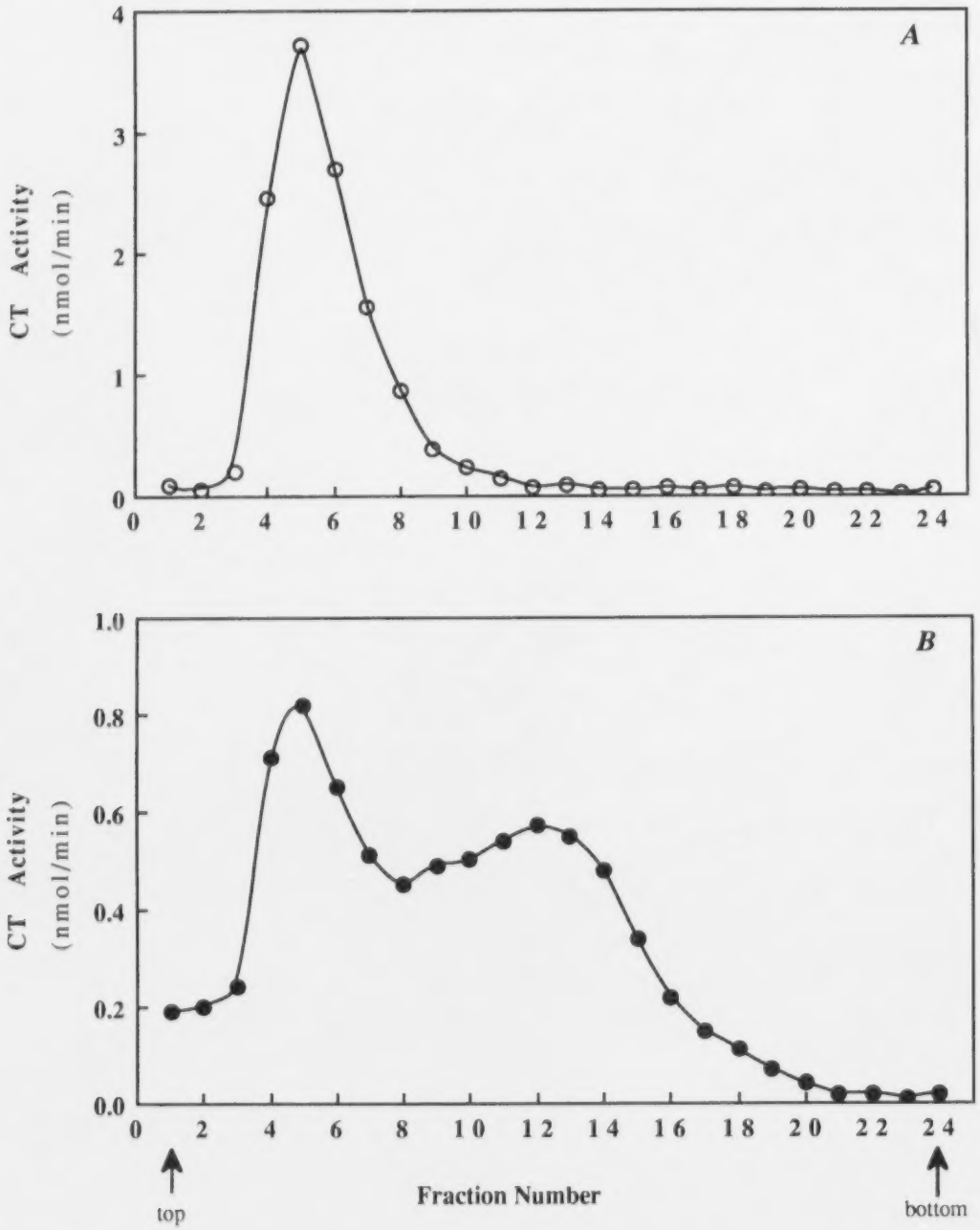

A
B
CT Activity
(nmol/min)
0.0
0.2
0.4
0.6
0.8
0 2 4 6 8 10 12 14 16 18 20 22 24
Fraction Number
top of
the tube
bottom of
the tube

Fig. 53. Separation of H and L Forms of Rat Liver and Rat Lung CT by Glycerol Gradient Centrifugation. Cytosolic samples from (*A*) rat liver (6.16 mg) or (*B*) rat lung (5.2 mg) were layered on 12 ml, 8 - 40% (v/v) linear glycerol gradients and centrifuged at 40,000 rpm for 14 h at 4°C. 0.5 ml aliquots were removed starting from the top of the tube and assayed for CT activity.


A
CT Activity
(nmol/min)
4
3
2
1
0
0 2 4 6 8 10 12 14 16 18 20 22 24
B
CT Activity
(nmol/min)
1.0
0.8
0.6
0.4
0.2
0.0
0 2 4 6 8 10 12 14 16 18 20 22 24
top
Fraction Number
bottom

distribution of CT. This mechanism was investigated in TPA-treated HeLa cells since there was evidence that TPA-treatment caused PC hydrolysis (Glatz *et al.*, 1987). The situation was complicated, however, because although TPA causes hydrolysis of PC there is also the formation of DG which has been shown elsewhere in the present study to translocate CT. Moreover, previous studies by Cornell and Vance (1987) have shown that DG translocated CT *in vitro*. Intracellular PC levels were unchanged in both TPA-treated cells (187 ± 12 nmol/mg membrane protein) and controls (184 ± 8 nmol/mg membrane protein) as were PE levels (42 ± 4 and 46 ± 3 nmol/mg membrane protein in controls and TPA-treated cells, respectively). This is in agreement with previous studies from this laboratory (Mueller, H.W. and Vance, D.E., unpublished observations).

As an approach to changing intracellular PC levels without affecting DG levels, experiments were attempted in which HeLa cells were placed in choline deficient medium for 24 h. The cells were then digitonin-permeabilized and CT activities in the cytosolic and particulate fractions were determined. The results from these experiments were largely inconclusive as PC levels were unchanged from cells in normal medium compared to cells in choline deficient medium. Subcellular distribution of CT activity also appeared to be unchanged.

3.5 THE ROLE OF THE CYTOSKELETON IN PC BIOSYNTHESIS AND CT TRANSLOCATION

There is increasing evidence in the literature that enzymes and intermediates of metabolic pathways are not freely diffusible within a cell but form highly organized and functional associations. Studies from the

laboratory of M.W. Spence indicate that the PC biosynthetic pathway in cultured glioma cells is highly organized, suggesting that the reaction intermediates are not freely diffusible within these cells but are channeled to PC biosynthesis (George *et al.*, 1989). For metabolic compartmentation to occur it is proposed in the present study that the cytoskeleton which is a highly organized structure itself would be involved in confering structure and organization to a metabolic pathway, specifically to the enzymes of the PC biosynthetic pathway. Therefore, studies were initiated to determine what role, if any, the cytoskeleton played in PC biosynthesis in HeLa cells. Several cytoskeletal disrupting agents were used to investigate the role of the cytoskeleton on PC biosynthesis in HeLa cells. Moreover, since TPA is known to cause the polymerization of actin (Rao, 1985; Keller *et al.*, 1985) which forms the microfilaments of the cytoskeleton, it was conceivable that the TPA-elicited translocation ocurred by the association of CT with actin. In addition, diacylglycerols such as OaG and DiC_8 also cause the polymerization of actin (Keller *et al.*, 1989) and, as demonstrated in section 3.3, cause CT translocation to membranes. Some support for this hypothesis was lent by Hunt *et al.* (1990), who postulated that CT in human and rat lung associated *in vitro* with actin. Although the evidence provided by Hunt *et al* (1990) for this association is largely circumstantial, the theoretical basis for the postulation appears to be sound.

3.5.1. *The Effect of Anti-Tubulin Agents on PC biosynthesis in HeLa Cells*

Anti-tubulin agents such as colchicine, vinblastine and nocodazole were used to disrupt microtubules. Colchicine, an alkaloid extracted from the meadow saffron binds to tubulin dimers inhibiting the addition of tubulin

molecules to microtubules thereby leading to microtubule depolymerization (Taylor, 1965; Inoue and Sato, 1967). Vinblastine inhibits microtubule formation by inducing the formation of paracrystalline aggregates of tubulin (Fugiwara and Tilney, 1975) and has been widely used as an anti-cancer drug. Nocodazole, a powerful inhibitor of mitosis, blocks microtubule assembly *in vitro* (Hoebeke *et al.*, 1976).

Initially, pulse-chase experiments were performed in the absence or presence of these microtubule-disrupting agents and their effects on PC biosynthesis were evaluated either when present by themselves or along with TPA. The goal was to study the effect of microtubule-disrupting agents on TPA-stimulated PC biosynthesis. HeLa cells were prelabeled with 2 μCi/ml [*methyl*-^{3}H]choline chloride for 1 h and chased in the absence or presence of the various microtubule-disrupting agents ± 100 nM TPA. After a 1 h chase cells were harvested, lipids extracted and the radiolabel in the choline-containing metabolites determined. The results are shown in Table 4. Colchicine, vinblastine and nocodazole had no effect on the incorporation of [^{3}H]choline into the total lipid fraction when present by themselves. Neither did any of the agents have any effect on TPA-stimulated PC biosynthesis. The concentrations of agents used in these studies were similar to those used in the literature to achieve maximal results.

Experiments were also conducted to determine if these agents had any effect on CT translocation in the presence of TPA or when present by themselves. The results were largely inconclusive as the method to disrupt cells employed in these studies was Dounce-homogenization. These experiments were conducted before the digitonin-permeabilization method was routinely utilized to study CT translocation.

Table 4. The Effect of Anti-Tubulin Agents on PC Biosynthesis. HeLa cells were prelabeled with 2 μCi/ml [*methyl*-^{3}H]choline chloride for 1 h. The labeled medium was replaced with unlabeled medium containing various tubulin-disrupting agents ± 100 nM TPA as shown. Controls were incubated in the presence of 0.2% DMSO, the highest concentration of DMSO present in any given sample. After 1 h cells were harvested, lipids extracted and the radioactivity in the choline-containing aqueous metabolites and in the total lipid fraction was determined by liquid scintillation counting. Unless indicated otherwise the values are averages of duplicate samples from two different experiments.

Table 4

Additions	Aqueous choline-containing metabolites (dpm x 10^{-6}/mg)	Total Lipids (dpm x 10^{-5}/mg)
0.2% DMSO (Control)	1.60 ± 0.04 (n=4)	0.75 ± 0.03 (n=4)
100 nM TPA	1.25 ± 0.10 (n=8)	2.24 ± 0.11 (n=8)
10 μM Colchicine	1.49	0.83
TPA + Colchicine	1.18	2.59
10 μM Vinblastine	1.53	0.68
TPA + Vinblastine	1.13	2.21
3 μM Nocodazole	1.53	0.84
TPA + Nocodazole	1.19	2.21

3.5.2. *The Effect of Cytochalasin E on TPA-Stimulated PC Biosynthesis and CT Translocation in HeLa Cells*

The cytochalasins (cytochalasin A, B, C, D and E) are a family of metabolites excreted by various species of moulds. They are employed as actin-binding drugs that inhibit the addition of actin molecules to actin filaments, leading to microfilament depolarization. Cytochalasin E has been described as the most potent of all the cytochalasins in inhibiting actin polymerization (Lin *et al.*, 1980). Therefore, cytochalasin E (CE) was chosen as the microfilament-disrupting agent in the present study.

(a) The effect of CE on PC biosynthesis - As a preliminary study to investigate the effect of microfilament disruption on TPA-stimulated PC biosynthesis, HeLa cells were prelabeled with 2 μCi/ml [*methyl*-^{3}H]choline chloride for 1 h. Subsequently, the cells were chased in the absence or presence of CE ± 100 nM TPA. After 1 h the cells were harvested, lipids extracted and the radiolabel in the choline-containing metabolites determined. 10 μM CE was used in initial studies. Experiments with various CE concentrations (0.1, 1, and 10 μM) revealed that 1 μM CE was as effective in enhancing TPA-stimulated PC biosynthesis as 10 μM CE. Therefore, 1 μM CE was used in subsequent experiments. A summary of the pulse-chase experiments is shown in Table 5. CE alone had no effect on the incorporation of [^{3}H]choline into the total lipid fraction. Surprisingly, instead of inhibiting TPA-stimulated incorporation of [^{3}H]choline into PC as would be expected if the TPA-effects were mediated by actin, CE *enhanced* the TPA-effect. The presence of 100 nM TPA alone increased the incorporation of [^{3}H]choline into the total lipid fraction by 3-fold in agreement with previous results in the present study from 0.75 ± 0.03 x 10^5 dpm/mg (in controls) to 2.24 ± 0.03 x 10^5 dpm/mg. The presence of 1 μM CE enhanced this significantly from 2.24 ± 0.03 to 4.35 ± 0.1 x

10^5 dpm/mg. This increase was statistically significant as indicated in Table 5. An analysis of the aqueous choline-containing metabolites revealed a decrease in the level of radiolabeled phosphochline, indicating an enhancement of the CT-catalyzed reaction. There was no difference in the levels of radiolabeled CDP-choline between cells incubated with TPA alone or with TPA + CE (4.41 and 4.02 x 10^3 dpm/mg, respectively). There was an approximately 2-fold increase in cellular [^{3}H]choline when TPA and CE were present together (8.0 ± 1.27 x 10^3 dpm/mg) compared to when TPA was present alone (4.31 ± 1.2 x 10^3 dpm/mg).

(b) The effect of CE on CT translocation - Since a reaction catalyzed by CT was implicated in the previous section, the effect of CE on TPA-elicited CT translocation to membranes was studied. Initial studies using Dounce-homogenization were largely inconclusive due to the large variations between samples and experiments. Digitonin-permeabilization experiments revealed interesting results as summarized in Table 6. HeLa cells were incubated in the absence or presence of CE ± 100 nM TPA for 1 h. Subsequently, the cells were permeabilied with digitonin for 5 min and the CT activities in the particulate fractions determined. Incubation of cells in the presence of CE alone increased membrane-associated CT activity from 0.27 in untreated controls to 0.45 nmol/min/mg. The TPA-elicited increase (0.74 nmol/min/mg) was enhanced to 1.10 nmol/min/mg when CE was present together with TPA.

Cytochalasins have been shown to increase intracellular calcium in various cell types (Treves *et al.*, 1986). In addition, work from this laboratory has shown that increasing intracellular Ca^{2+} in rat hepatocytes with the help of the ionophore A23187 increased PC biosynthesis as well as CT translocation

Table 5. The Distribution of [^{3}H]choline in Choline-Containing Metabolites in Cells Treated with Cytochalasin E ± TPA. HeLa cells were prelabeled for 1 h with 2 μCi/ml [*methyl*-^{3}H]choline chloride for 1 h. The labeled medium was replaced with unlabeled medium ± agents as shown. Controls were incubated in the presence of 0.2% DMSO, the highest concentration of DMSO present in any given sample. After 1 h the cells were harvested and lipids extracted. Aqueous metabolites were separated by TLC in methanol : 1.2% NaCl : NH_4OH (10 : 10 : 1). In this solvent system choline, phosphocholine, glycerophosphocholine and CDP-choline exhibited R_F values of 0.15, 0.38, 0.51 and 0.59, respectively. The separated aqueous metabolites were scraped off the TLC plates and subjected to liquid scintillation counting. The radioactivity in the total lipid fraction was also determined. Unless indicated otherwise the results are averages of duplicate samples from two different experiments.

* $0.005 \leq p \leq 0.01$

Table 5

Additions	Aqueous choline-containing metabolites: choline (dpm x 10^{-3}/mg)	phospho-choline (dpm x 10^{-6} /mg)	CDP-choline (dpm x 10^{-3}/mg)	GPC (dpm x 10^{-3} /gm)	Total Lipids (dpm x 10^{-5}/mg)
I. Control (0.2% DMSO)	3.19 ± 0.08 (n=3)	1.08 ± 0.02 (n=3)	3.38	0.87	0.75 ± 0.03 (n=8)
II. TPA (100 nM)	4.31 ± 1.20 (n=3)	0.86	4.41	1.25	2.24 ± 0.03 (n=8)
III. 1 μM CE	3.33	0.99	2.74	1.0	0.74 ± 0.06 (n=4)
IV. TPA + CE	8.06 ± 1.27 (n=4)	0.68 ± 1.27 (n=4)	4.02 ± 0.52 (n=4)	1.44	4.35 ± 0.10 (n=4) II vs IV*

to membranes (Sanghera and Vance, 1989). The study also showed that these effects could be partially overcome by Verapamil, a calcium channel blocker. In order to determine whether calcium was directly involved in the association of CT with membranes after CE treatment of HeLa cells, digitonin-permeabilization of cells was performed in the presence of 5 mM EGTA after the cells had been treated with the various agents (Table 6). The inclusion of EGTA appeared to have eliminated the CE-mediated increase in membrane-associated CT acitivity (0.392 nmol/min/mg in CE-treated cells compared to 0.353 nmol/min/mg in untreated controls). However, EGTA did not appear to affect the increase in membrane-associated CT seen when TPA and CE were present together. To find out whether the presence of EGTA was affecting CT activity by removing Ca^{2+} that might be inhibiting CT activity, particulate CT assays were performed ± 5 mM EGTA. There was no difference in CT activities in the presence or absence of EGTA (data not shown).

(c) The effect of CE on cellular DG levels - Since the cellular levels of DG are important in the translocation of CT (sections 3.2 and 3.3), and can limit PC biosynthesis (Jamil *et al.*, 1992) experiments to determine DG mass in CE-treated cells were performed.

HeLa cells were incubated in the absence or presence of 1 μM CE ± 100 nM TPA for 1 h. Subsequently, the cells were harvested, lipids extracted and the DG quantitated by the DG kinase method described in section 2.15.2. Fig. 54 shows the results of such an experiment. As expected the presence of TPA increased the DG levels from 1.8 nmol/mg in controls (lane 1) to 3.67 nmol/mg (lane 2). Incubation of cells with 1 μM CE for 1 h showed a small increase over controls to 2.37 nmol/mg (lane 3). Interestingly, incubation of cells with TPAand CE together decreased the TPA-elicited DG increase to 3.33 nmol/mg (lane 4).

Table 6. The Effect of Cytochalasin E ± TPA on the Amount of Membrane CT in HeLa Cells. HeLa cells were incubated in the presence of agents for 1 h as shown. Controls were incubated in the presence of 0.2% DMSO, the highest concentration present in any given sample. Subsequently the cells were digitonin-permeabilized for 5 min in the absence or presence of 5 mM EGTA. Specific CT activities were determined in the particulate fractions and the results are presented as the average values of duplicate samples from two separate experiments.

	Particulate CT Activity (nmol/min/mg)	
Additions	minus EGTA[a]	plus 5 mM EGTA[b]
Control (0.2% DMSO)	0.269	0.353
TPA	0.740	0.925
Cytochalasin E	0.446	0.392
TPA + Cytochalasin E	1.102	1.14

a cells were digitonin-permeabilized in the absence of EGTA
b cells were digitonin-permeabilized in the presence of 5 mM EGTA

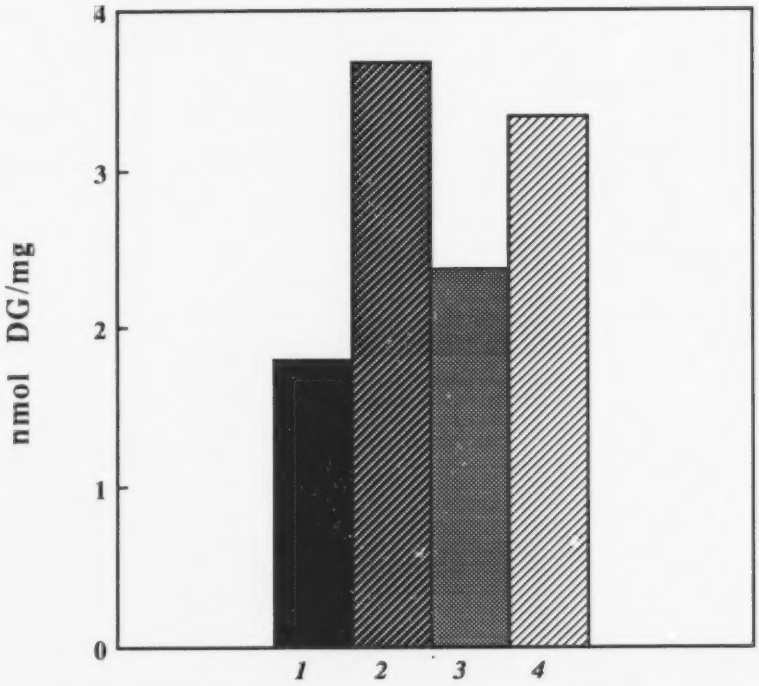


Fig. 54. The Effect of Cytochalasin E ± TPA on Intracellular DG Levels. HeLa cells were incubated in the absence (lane 1) or presence of 100 nM TPA (lane 2), 1 μM CE (lane 3), and TPA + CE (lane 4) for 1 h. Subsequently the cells were harvested, lipids extracted according to the method of Bligh and Dyer (1959) and DG levels quantitated by the DG kinase method as described in section 2.15.2. Untreated controls (lane 1) were incubated in the presence of 0.2% DMSO, the highest level of DMSO present in any given sample. The results are expressed as nmol DG/mg protein.

3.6. IMMUNOFLUORESCENT STUDIES ON CT

CT appears to exist in a dual location in many cell types - in the cytosol and associated with membranes. Originally, Wilgram and Kennedy (1963) found that cytosolic and microsomal CT activities were present in roughly equal amounts in the rat liver. In embryonic muscle cells, cytosolic CT activity was shown to be 78% of the total activity present (Sleight and Kent, 1980). In HeLa cells, digitonin-permeabilization released 97% of total cellular activity and only 3% remained associated with membranes, as seen in previous sections in the present study.

Although PC biosynthesis has been reported to occur in several organelles in animal cells the major quantitative site appears to be the endoplasmic reticulum (ER). The location of CT was first described by Wilgram and Kennedy (1963) to be microsomal in rat liver. Subsequently, studies from two different laboratories localized CT more specifically to the ER (Tercé *et al.*, 1988; Vance and Vance, 1988). Vance and Vance (1988) also reported CT activities associated with the *cis*-golgi. Morand and Kent (1989) reported a nuclear-envelope location for CT in CHO cells that had an altered membrane composition. Functionally it would appear more reasonable for CT to be associated with the ER, along with other enzymes of the PC biosynthetic pathway, notably, cholinephosphotransferase.

With the availability of an antibody to CT it would be possible to unequivocally demonstrate the subcellular location of CT with the help of electron microscopy. The aim was to investigate whether CT was indeed functionally located at the ER and if so, what particular region of the ER. Another aim was to investigate, if possible, the claim by Hunt *et al.* (1990) that

CT associates with actin and, by extrapolation, with microfilaments of the cytoskeleton.

3.6.1. *Immunofluorescent Studies on CT in Intact HeLa Cells*

Initial studies were done using immunofluorescence techniques on whole cells. The objective was to see if CT could be detected in cells before further experiments were performed on the detection and location of membrane-associated CT.

HeLa cells growing on glass slides were fixed to the slides and the detection and visualization of CT was performed according to the procedure described in section 2.20. Positive and negative controls were included in the procedure. Fig. 55 shows the results of such an experiment. Panel A shows cells that had been incubated with a rabbit antibody to a synthetic peptide corresponding to an N-terminal region of rat liver CT. In panel B cells were incubated with non-specific IgG from immune serum, while panel C shows a positive control in which cells were incubated with a rabbit antibody to actin. The results of the negative control (minus primary antibody) are shown in panel D.

3.6.2. *Detection of Membrane-Associated CT by Immunofluorescence*

It appeared from Fig. 55 A that CT could be detected easily by immunofluorescent techniques. The next step was to visualize membrane-associated CT which was done by first releasing cytosolic CT by digitonin-permeabilization followed by fixing the cell ghosts to the glass slides and probing with the antibody to CT. The cells were treated with TPA or oleate so that increases in membrane CT due to these treatments could be detected. The results are shown in Fig. 56. HeLa cells growing on 4-chamber glass slides

were treated in the absence (panel A) or presence (panel B) of 100 nM TPA for 30 min, 0.35 mM oleate for 30 min (panel C) and 0.35 mM oleate for 30 min followed by 10 mg/ml albumin for a further 30 min (panel D). At the end of these treatments the cells were permeabilized with digitonin for 5 min to release the cytosolic CT, followed by fixing the resulting cell-ghosts to the glass slides. The cells were probed with antibody to CT and visualized as described in section 2.21. There did not appear to be any difference in the fluorescent intensity emitted from the cells as a result of these manipulations.

Immunofluorescence studies were also done on HeLa cells on another slide where after digitonin-permeabilization the cell gosts were fixed and probed with non-CT antibodies as shown in Fig. 57. The cells shown in panel A were probed with non-specific IgG from immune serum, whereas the cells in panel B were probed with rabbit antibodies to actin and panel C was the negative control where no primary antibody was used.

Two interpretations could be drawn from the above results:

i. The fluorescence seen in these slides was not due to CT but some other antigen cross-reacting strongly with the antibody to CT

ii. The cells were in an active log phase and, therefore, the majority of CT would be found associated with membranes to facilitate the synthesis of PC since increased amounts of PC would be required in actively growing cells.

To test the second hypothesis, the subcellular distribution of CT activities were examined at different growth stages of HeLa cells as described in the following section.

Fig. 55. Detection of CT in Intact HeLa Cells by Indirect Immunofluorescence. Intact HeLa cells fixed on glass slides were probed with a rabbit antibody to CT (panel A), a non-specific antibody (panel B), a rabbit antibody to α-actin (panel C) and minus primary antibody (panel D) as described in section 2.24. The cells were visualized by indirect immunofluorescence. Bar, 1 μm.

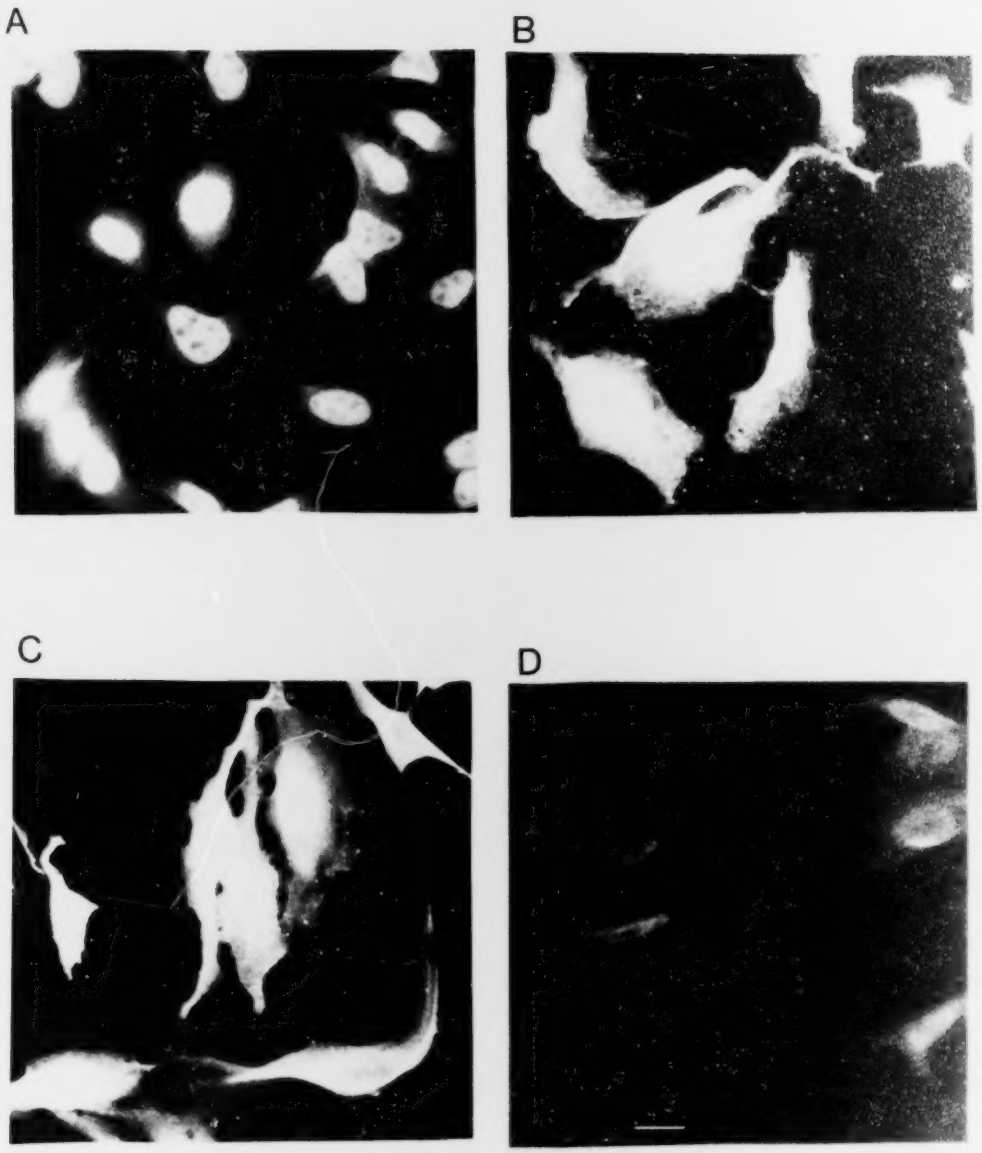

A
B
C
D

Fig. 56. Detection of Membrane-Associated CT by Indirect Immunofluorescence in Digitonin-Permeabilized HeLa Cells. HeLa cells were incubated in the absence (panel A) or presence of 100 nM TPA for 30 min (panel B), 0.35 mM Oleate for 60 min (panel C) and 0.35 mM oleate for 30 min followed by the addition of 10 mg/ml albumin for a further 30 min (panel D). Following these treatments the cells were permeabilized with digitonin, fixed and probed with a rabbit antibody to CT. The cells were visualized by indirect immunofluorescence as described in section 2.24. Bar, 1 μm.

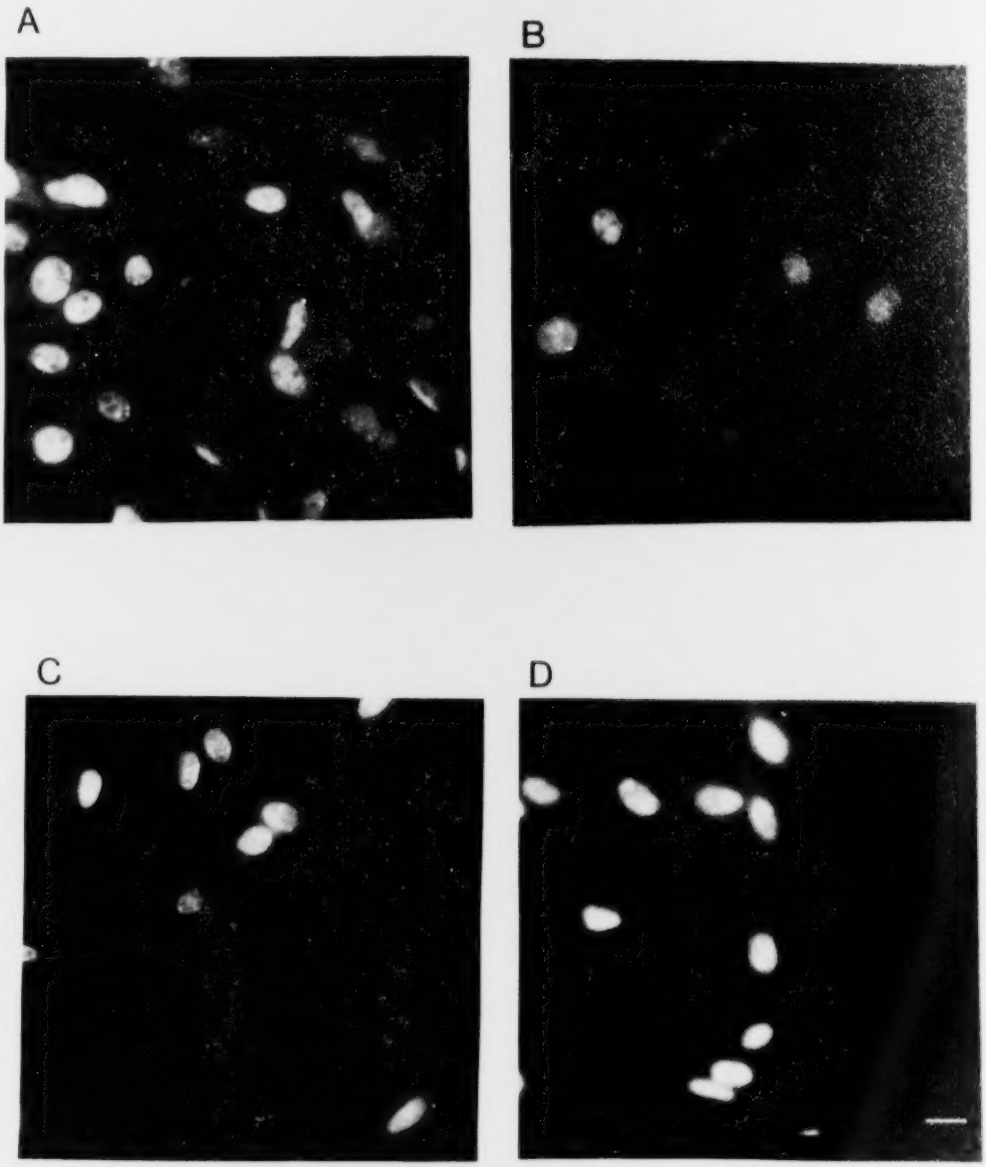
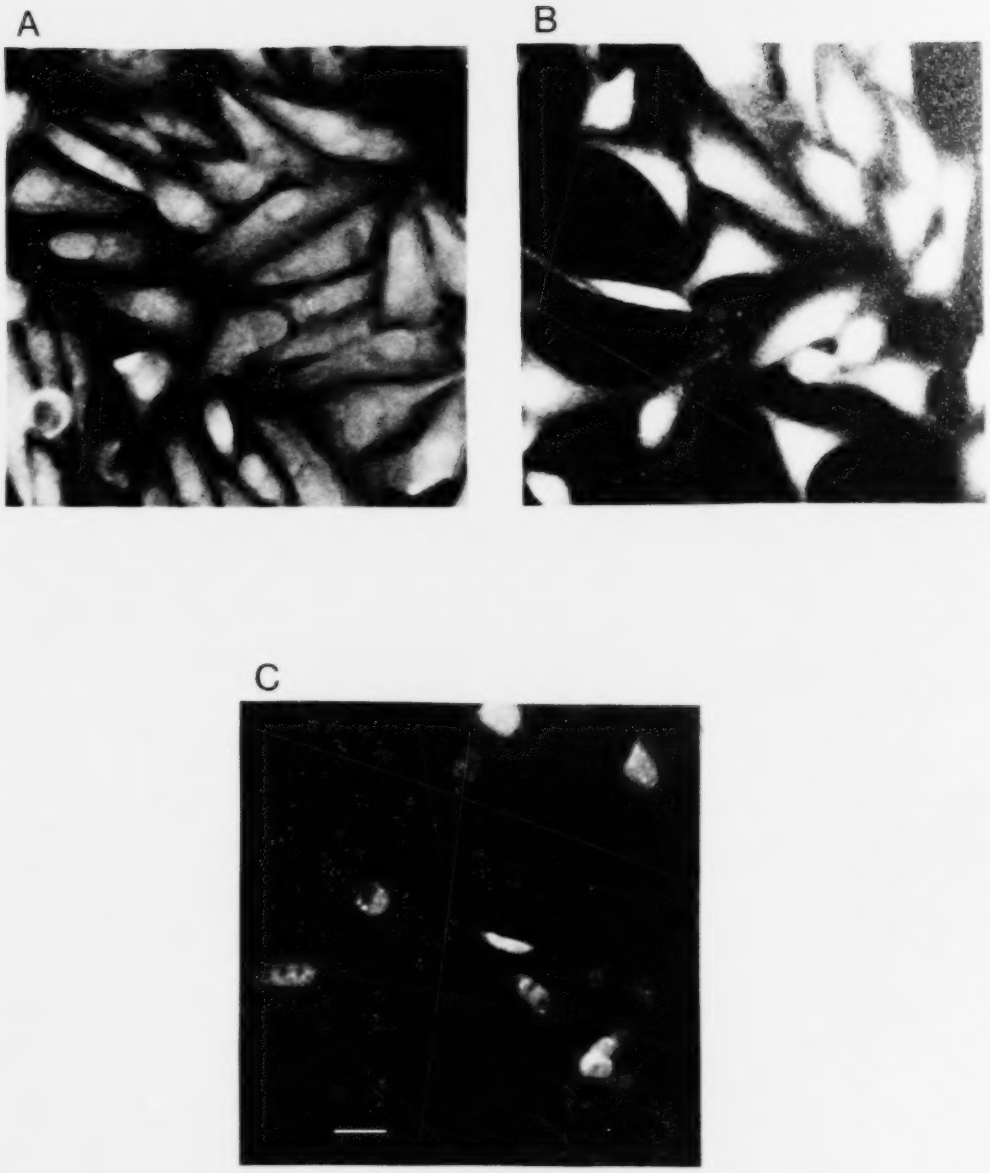

A
B
C
D

Fig. 57. Detection of Non-CT Antigens by Immunofluorescence.

HeLa cells were digitonin-permeabilized and probed with a non-specific antibody to CT (panel A), a rabbit antibody to α-actin (panel B) and minus primary antibody (panel C). The cells were visualized by indirect immunofluorescence. Bar, 1 μm.


A
B
C

3.6.3. *The Effect of Growth Phase on the Subcellular Distribution of CT*

HeLa cells were plated in 60 mm dishes at a low density (10^5 cells/dish) so that two sets of dishes had cells in an active log phase. One set of dishes was plated at twice the density of the other dishes so that at the time of the experiment the cells were fully confluent. One set of dishes that had cells in an active log phase were put in serum-free medium for 24 h before the experiment to stop their growth and make them quiescent. All three sets of cells were digitonin-permeabilized and the CT activities in the cytosolic and particulate fractions determined. The results are shown in Table 7. It was expected that cells in the log phase would have higher membrane-associated CT activities than cells that were growth-arrested or were confluent. However, as can be seen from Table 7 there appeared to be no obvious differences among membrane-associated CT activities from cells at different stages of growth.

Therefore, from the results of these experiments it was concluded that the antigen detected by the rabbit antibody to CT was not CT but another protein that was not released during digitonin treatment and that cross-reacted with the antibody giving a strong signal in the immunofluorescence studies. As a result of this reasoning the immunocytochemical studies on CT were not carried to their logical conclusion, that of electron microscopy.

Table 7. Subcellular Distribution of CT in HeLa Cells at Different Stages of Growth. HeLa cells were plated at a low density (10^5 cells /dish) in two sets of 60 mm dishes (I and II). The cells in set I were placed in serum-free medium for 24 h prior to the experiment, whereas the cells in set II were left in an actively growing state in growth medium. The dishes in set III were plated at twice the density in either sets I or II so that the cells were fully confluent at the time of the experiment. Cells were digitonin-permeabilized for 5 min and specific CT activities in the released cytosol and retained particulate fractions determined. The results shown are averages of duplicate samples from two separate experiments.

	CT Activity (nmol/min/mg)	
	Cytosol	Particulate
I. Growth-arrested (quiescent) cells	6.69	0.350
II. Actively-growing cells	7.27	0.368
II. Confluent cells	7.11	0.425

CHAPTER 4 DISCUSSION

4.1. THE STIMULATION OF PC BIOSYNTHESIS BY TPA

In section 2.1 it was shown by pulse-chase experiments that TPA stimulates the biosynthesis of PC 3-4 fold in HeLa cells. This is in agreement with previous reports. Kinzel *et al.* (1979) showed that 100 nM TPA increased the labeling of choline-containing lipids in Hela cells. Subsequently, it was shown in pulse-chase experiments that the stimulation of PC labeling was accompanied by a decrease in label in the aqueous precursor, phosphocholine (Paddon and Vance, 1980). This suggested that the accelerated disappearence of labeled phosphocholine in TPA-stimulated cells was due to its rapid conversion to CDP-choline and subsequent incorporation into PC. Since CDP-choline constituted less than 5% of the labeled aqueous metabolites it was suggested that the stimulation of PC biosynthesis was due to the stimulation of the CT-catalyzed reaction. It was also shown that whereas the other enzymes of the CDP-choline pathway, choline kinase and cholinephosphotransferase were unaffected by TPA-treatment, CT activity was increased. CT activity appeared to be activated by a redistribution from an inactive cytosolic form to an active membrane-associated form (Pelech, Paddon and Vance, 1984).

In the present study the TPA-stimulated PC biosynthesis via CT was confirmed and CT translocation to membranes was demonstrated both by measuring activity and by immunoblot analyses (section 2.1).

4.1.1. *TPA-Elicited Translocation of CT Activity to Membranes*

Of the three methods used to rupture cells to obtain subcellular fractions digitonin-mediated release of cytosolic contents provided information about the distribution of CT that best explained the results of the pulse-chase

experiments. The Dounce homogenization method of rupturing cells showed that 82% of the total activity was present in the cytosolic fraction and 18% in the microsomal fraction. Upon 30 min of TPA treatment membrane-associated CT activity increased to 33% and the cytosolic activity decreased to 67% of the total cellular activity (Fig. 12).

On the other hand the nitrogen cavitation experiments indicated that both cytosolic and microsomal CT activities increased after TPA-treatment (Fig. 14) and indicated a lack of CT translocation to membranes (Table 1).

The Dounce homogenization experiments showed that approximately 20% of total CT activity was present in microsomes whereas the digitonin-permeabilization experiments showed that only 3% of the total CT activity was membrane-associated. The 3-fold stimulation of PC biosynthesis by TPA suggested a CT activation of similar magnitude particularly as the *in vitro* activity of CT and its Km for CPT were unchanged (Paddon and Vance, 1980). The digitonin-permeabilization experiments showed that indeed TPA-treatment resulted in a 3-fold increase in membrane-associated CT activity. This was confirmed by immunoblotting experiments.

The findings in this study were directly contradicted by Watkins and Kent (1990) who reported that although a stimulation of PC biosynthesis was seen in TPA-treated HeLa cells there was no decrease in cytosolic CT activity. Watkins and Kent interpreted this to mean a lack of CT translocation to membranes and suggested that TPA-stimulated PC biosynthesis via CT occurred by a mechanism other than increased association with membranes perhaps a reversible phosphorylation mechanism. However, their studies showed that the phosphorylation state of cytosolic CT was unchanged in control and TPA-treated cells. There appear to be two salient points that were overlooked by these authors:

1) Due to the relatively large pool of cytosolic CT (97% of total CT activity as seen by digitonin-mediated release), redistribution of a fraction of this activity to membranes would not be apparent beyond experimental error. Moreover, the data were reported as % of total activity and actual values were not reported.

2) The authors did not report membrane-associated CT activities after digitonin-mediated release of the cytosolic contents. This was critical to the interpretations drawn from the study. A minor loss of CT activity (0.11 nmol/min/dish) from a relatively large cytosolic pool (1.24 nmol/min/dish in control cells) translated into a significantly large increase in membrane-associated CT activity (0.04 nmol/min in control cells to 0.15 nmol/min after TPA-treatment).

Dounce homogenization experiments showed only a 1.5-fold increase in microsomal CT activity in TPA-treated cells (4.43 nmol/min/mg) compared to controls (6.43 nmol/min/mg) after 30 min. Since the homogenization procedure itself can be expected to produce vesicles and alter the subcellular distribution of CT in favour of microsomes, it is possible that the extent of CT translocation seen after TPA-treatment was underestimated. If the microsomal activity were high to start with an increase in CT activity that would be three times the *true* microsomal activity would not be three times the *apparent* (higher than the true value) microsomal activity. Therefore, the increase in microsomal CT activity in TPA-treated cells appears to be only 1.5 times that in controls.

The reason for the lack of translocation after TPA-treatment in the nitrogen cavitation experiments is unclear. It is possible that the high pressures of nitrogen used in these studies produce artifacts that cause changes in the enzyme itself.

Digitonin-permeabilization of cells, the least destructive method for rupturing cells, gave results in terms of CT translocation that could best account for the changes seen in PC biosynthesis upon TPA-treatment. Moreover, the utility of this method was further demonstrated in oleate-elicited CT translocation (Fig. 18) and in REF 52 cells, another cell-line whose PC biosynthesis is stimulated by TPA via CT activation. Dounce homogenization of these cells to study subcellular distribution of CT gave inconclusive results. Digitonin-permeabilization showed that TPA causes a decrease in cytosolic CT activity accompanied by an increase in membrane-associated activity (Fig. 20). The digitonin-permeabilization method, therefore, provides information on the subcellular distribution of CT that best agrees with observed changes in PC biosynthesis by TPA.

4.1.2. *Confirmation of CT Translocation by Immunoblotting*

It was imperative to show that the increase in membrane-associated CT activity was due to an increase in the amount of the enzyme itself and not due to changes in activity. This was done by immunoblot analyses. Initial attempts to visualize translocation using ^{125}I-protein A for detection on immunoblots were only partially successful as cytosolic CT could be detected but not membrane-associated CT (Fig. 22). Since the membrane-associated CT constituted only 3% of the total activity this was not surprising and more sensitive means of detection were employed. A recently available technique that utilizes enhanced chemiluminescence of horseradish peroxidase-catalyzed H_20_2 hydrolysis (the principle of the method is described in Appendix B) was successfully used to detect membrane-associated CT. By this method an increase in membrane-associated CT in TPA-treated HeLa cells was observed, thus confirming that translocation of the enzyme had indeed taken

place (Fig. 23). CT translocation by oleate and its reversal by albumin was also demonstrated by immunoblotting (Fig. 24)

4.2. THE ROLE OF DG AS A TRANSLOCATION SIGNAL FOR CT

There are several indications in the literature that implicate DG as a translocation signal for CT. Treatment of various cell-types with TPA has been shown to increase the formation of DG (Daniel *et al.*, 1986; Besterman *et al.*, 1986; Glatz, *et al.*, 1987; Cabot *et al.*, 1988). In addition, Cornell and Vance (1987a) demonstrated enhanced binding of CT to DG-enriched vesicles. More recent evidence from Kolesnick and Hermer (1990) showed that DiC_8, a DG analogue, caused purified CT to translocate to boiled microsomes.

4.2.1. *DG as a Mediator of TPA-Elicited CT Translocation*

In the present study the role of DG in signaling translocation of CT to membranes was investigated. As a preliminary study towards evaluating this role of DG, [^{3}H]DG was shown to accumulate in HeLa cells prelabeled with [2-^{3}H]glycerol and chased in the absence or presence of TPA (Figs. 26 and 27). Although Glatz *et al.* (1987) have shown that the increased DG upon TPA-treatment in HeLa cells is derived from PC, no apparent decrease in [^{3}H]PC in TPA-treated cells was seen in Fig. 27. This is not surprising because the [^{3}H]PC had more than 100 times more radioactivity than DG and a difference of 0.035 x 10^6 dpm/mg in [^{3}H]DG between controls and TPA-treated cells would not be apparent as a decrease in [^{3}H]PC in TPA-treated cells.

From the time-course of [^{3}H]DG accumulation (Fig. 26) it was apparent that the [^{3}H]DG in control cells was rapidly metabolized and that the level of [^{3}H]DG seen in TPA-treated cells was not seen as an *increase* but as two-fold more than in control cells. This likely represented a net balance between metabolism of

DG and its formation as a result of PC hydrolysis. An alternative explanation would be decreased turnover of DG allowing [^{3}H]DG to accumulate in TPA-treated cells. This appears to be unlikely in light of the report by Glatz *et al.* (1987) in which NBD-labeled DG was formed after treatment with TPA in Hela cells prelabeled with NBD-PC (a fluorescent analogue of PC) vesicles.

The increased DG in TPA-treated cells has been shown to be derived from PC hydrolysis, and not from increased *de novo* synthesis (see section 1.4.1). However, since it is not clear whether fatty acids which are precursors of DG were included in the incubation media of the cells under investigation, it is possible that *de novo* synthesis of DG was limited by fatty acid availability.

DG mass was measured in TPA-treated cells to show that cellular levels of DG were actually increased by TPA. This was greatly facilitated by a sensitive method for measuring DG which utilizes the quantitative conversion of DG to PA in the presence of DG kinase and [γ-^{32}P]ATP (Priess *et al.*, 1986; Wright *et al.*, 1988). As expected, TPA caused cellular DG levels to increase from 2.29 nmol/mg to 4.02 $\pm$ 0.7 nmol/mg (Fig. 29). An investigation of the time-course of TPA-induced DG increase is consistent with a recent report by Van Blitterswijk *et al.*, (1991a) in which the earliest significant increase in fatty-acid labeled DG in human fibroblasts was observed at 10 min after TPA-treatment.

Upon comparison of the time-course of DG accumulation (Fig. 30), CT translocation (Fig. 21) and PC biosynthesis (Fig. 11) it was possible to draw a temporal relationship among them. DG, which was maximally increased at 15 min of TPA-treatment appeared to signal the translocation of CT to membranes. At 15 min a partial CT translocation was seen which was maximal at 30 min. After a lag of 30 min PC biosynthesis was activated. These data indicate a temporal relationship among DG accumulation, CT translocation and PC

biosynthesis in which production of DG appeared to signal CT translocation which then increased the rate of PC biosynthesis.

The situation is complicated, however, because TPA-induced DG accumulation is a consequence of PC breakdown, which is the result of a putative phospholipase C-like reaction activated by PKC (Daniel *et al.*, 1986; Glatz *et al.*, 1987). Consequently, PC levels may have been decreased in TPA-treated cells. It is also known that decreased PC levels can increase the amount of membrane-associated CT (Yao *et al.*, 1990; Jamil *et al.*, 1990). There was no decrease in the cellular PC content, however, upon TPA treatment (184 ± 8 in controls compared to 187 ± 12 nmol/mg membrane protein in cells treated with TPA for 1 h) consistent with previous studies from this laboratory (Mueller, H.W. and Vance, D.E., unpublished observations).

To further evaluate the role of DG in CT translocation and hence PC biosynthesis an attempt was made to increase intracellular levels of DG by incubating cells with an exgenous source of DG. For this purpose a cell-permeant DG analogue, DiC_8, was used. OaG was not as effective as DiC_8 in causing CT translocation or in stimulating PC biosynthesis perhaps due to the relative instability of OaG. Indeed, OaG has been shown to be ineffective in causing the differentiation of HL60 cells compared to TPA and other stable DG analogues because of its metabolic instability in these cells. OaG also undergoes rapid isomerization to the 1,3-configuration, and when supplied to cells at high concentrations, modifies the fatty acid composition of cellular lipids (Welsh and Cabot, 1987). DiC_8 has been used extensively in a variety of cell systems as a source of exogenous DG (Kolesnick and Paley, 1987; Liscovitch *et al.*, 1987; Kolesnick, 1989). DiC_8 is, however, a known PKC activator in intact cells (Lapetina *et al.*, 1985). Since the objective was to increase DG without stimulating PC breakdown, it was necessary to bypass PKC activation. To

achieve this objective, PKC in HeLa cells was downregulated by treating the cells for 24 h with 1 μM TPA as seen by the immunoblot analyses in Figs. 31 and 32. Treatment of downregulated cells with various DGs of which DiC_8 was the most potent, caused the translocation of CT and stimulation of PC biosynthesis (Fig. 33 and 34). The presence or absence of TPA along with DiC_8 had no effect on either CT translocation or PC biosynthesis (Figs. 35 and 36) indicating that the effects were independent of PKC and entirely due to DiC_8.

In addition, the lag in CT translocation observed in TPA-treated cells (Fig. 21) was eliminated when the time-course of DiC_8-elicited CT translocation was examined in PKC-downregulated cells (Fig. 37). A comparison between DiC_8 accumulation (Fig. 40) and DiC_8-induced CT translocation (Fig. 37) revealed that changes in DiC_8 levels were closely followed by CT translocation.

These studies provide conclusive evidence for a role of DG in CT translocation. DG appears to cause directly CT to translocate to membranes, whether by directly interacting with CT or by making membranes more hydrophobic. Increased levels of DG, such as those seen in agonist- or TPA-stimulated cells, can significantly alter the physical state of membranes (Siegel *et al.*, 1989). As such changes in the membranes can decrease the radius of curvature of the lipid/water interphase (i.e. an increase in curvature) it is possible that more of the membrane is accessible with which CT can intercalate. Highly curved lipid vesicles are important for the intercalation of CT (Cornell, 1991b). A high degree of curvature indicates looser packing in the head group region, thereby facilitating intercalation of the protein between the lipids. CT has an amphipathic helix (Kalmar *et al.*, 1990) which may facilitate such an intercalation. Direct interaction between DG and CT cannot be eliminated. CT contains a domain near its amino terminus that has some homology with a conserved PKC amino acid sequence important

for phorbol ester or DG binding (Kalmar *et al.*, 1990). Phorbol esters themselves do not cause CT translocation *in vitro* (Cornell and Vance, 1987a; Kolesnick and Hermer, 1990).

There is evidence from the laboratory of Dr. Rosemary Cornell (Simon Fraser University) for a DG-responsive region at the COOH-terminus of rat liver CT. A comparison of the lipid regulation of the yeast and rat CT shows that yeast CT, which lacks the COOH-terminal amphipathic helix present in rat CT, does not bind to and is not activated by DG-enriched membranes (Cornell, R., Personal Communication).

4.2.2. *Is TPA- or DiC_8-Stimulated PC Biosynthesis Due to Increased Availability of Substrate?*

The availability of DG can, under certain conditions, regulate PC biosynthesis (Lim *et al.*, 1986) via the cholinephosphotransferase (CPT) reaction. It was suggested by these authors that since the membrane concentration of DG is well below its Km value, DG may be a potentially important regulator of PC biosynthesis in vivo (assuming an apparent Km value of CPT for DG as 28.5 mM, which is the value reported in lung microsomes by Ide and Weinhold, 1982). However, Lim *et al.* (1986) further suggested that the relatively small pool of CDP-choline of 30 μM (Pelech and Vance, 1984) compared to 230 μM DG, made CDP-choline a more likely contender for the role of a regulatory substrate than DG. Moreover, the results of the time course studies in the present study suggested that it is the increased production of CDP-choline (i.e., increased CT activity) that initially stimulated PC biosynthesis rather than DG. However, once PC biosynthesis is stimulated it is conceivable that an increased supply of DG would play an important role in maintaining the increased biosynthetic rate of PC, coordinately with the

increased conversion of phosphocholine to CDP-choline. The incorporation of DiC_8 into PC (section 3.3.7) suggests that DiC_8 was at the correct intracellular site for incorporation by CPT.

4.2.3. *Implications for the Involvement of DG in CT Translocation*

The implications for the involvement of DG in CT translocation as shown in this study are profound from two points of view:

1) DG, a product of PC hydrolysis, acts to stimulate PC synthesis by not only stimulating the regulatory enzyme i.e., CT but itself acting as a substrate for the CPT reaction, the final step in PC synthesis. The importance of DG as a regulatory substrate has been elucidated by Jamil *et al.* (1992), who have recently shown that the inhibition of PC biosynthesis when rat hepatocytes were treated with stable cAMP analogues is due to decreased availability of DG for the CPT reaction. This dual role of DG in the regulation of PC biosynthesis is illustrated in a proposed model (Fig. 58).

2) The results in this thesis can account for some unexplained observations in the literature, for example, previous studies have shown that phorbol esters and diacylglycerols stimulate PC biosynthesis by more than one pathway; one that is PKC-dependent and another that is PKC-independent (Liscovitch *et al.*, 1987; Kolesnick and Paley, 1987; Kolesnick, 1990). The PKC-dependent pathway appears to be activated by phorbol esters and low concentrations of DiC_8 of approximately 10-60 μM (Kolesnick, 1990), while the PKC-independent pathway is active at high concentrations of DiC_8 (approximately 500 μM, the concentration used in this study). Taken together with the results in the presenty study, these reports indicate that high concentrations of diacylglycerols act directly by modulating binding of CT to membranes, thereby stimulating PC biosynthesis. The finding that the two pathways are

additive (Liscovitch *et al.*, 1987; Kolesnick and Paley, 1987) can be explained by increased CT translocation - low concentrations of DiC_8 would activate PKC, resulting in an amplification of the signal for CT, namely DG, and at high concentrations DiC_8 itself would provide a signal for CT translocation to membranes.

Often DiC_8-mediated events on PC biosynthesis are described as being PKC-dependent or -independent and are, therefore, ascribed to different mechanisms or pathways. This is a simplistic view. It appears that far from being different pathways, there is a common mediator (CT) of these effects.

Changes in phospholipid metabolism due to high or low concentrations of exogenous DGs are not the only phenomena described as involving two pathways. Superoxide release by DiC_8-treated neutrophils can be inhibited by a PKC inhibitor at low but not high levels of DiC_8 (Badwey *et al.*, 1989). It is often overlooked that PKC activation, in general, results in the accumulation of DG (although there may be exceptions, as listed in a review by Kiss, 1990), and the DiC_8-mediated so-called 'PKC-independent' effects are merely the results of DG action i.e., providing the cells with DG itself. Bypassing PKC does not mean a different pathway as the words 'PKC-independent' imply.

4.2.4. *PC Mass does not Increase when PC Biosynthesis is Stimulated*

It is generally believed that a stimulation of PC biosynthesis is triggered by PC hydrolysis. This appears to be true in the present study as the increase in DG can be attributed to PC hydrolysis and the second messenger produced is then able to activate the CT reaction and stimulate PC biosynthesis. DiC_8-stimulated CT translocation and PC biosynthesis can be regarded as being

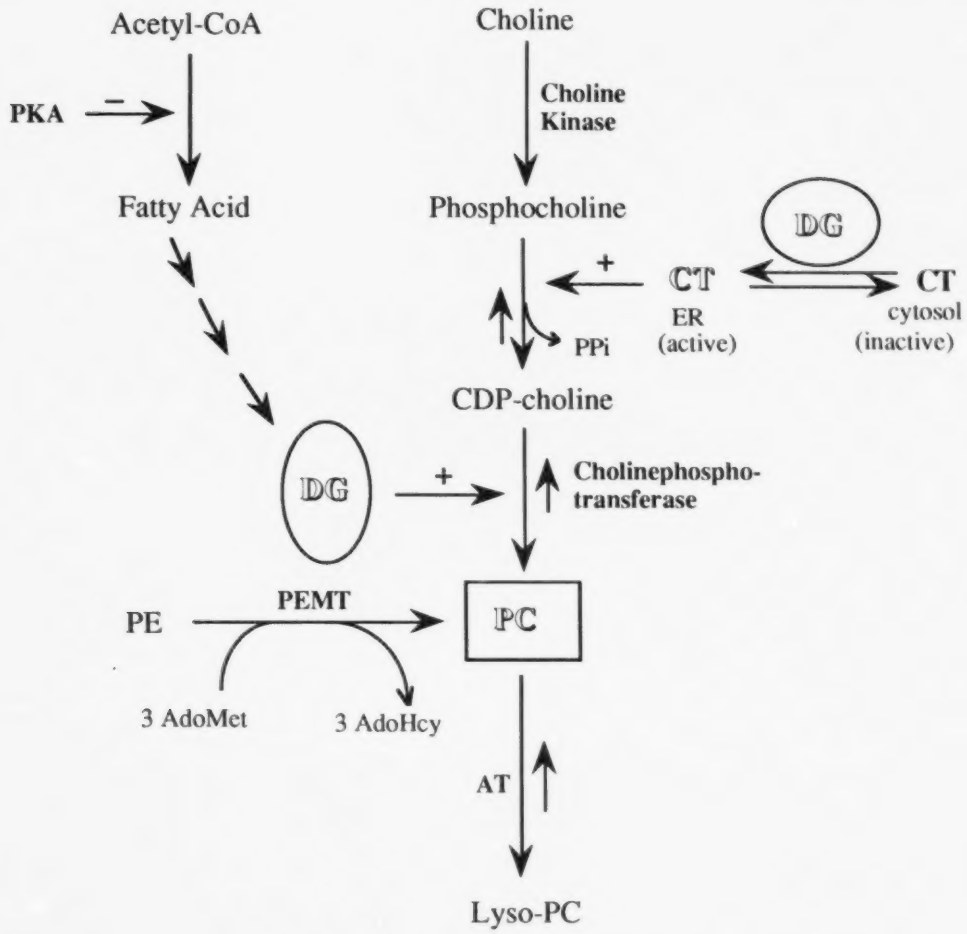


Fig. 58. Proposed Dual Role for Modulation of PC Biosynthesis by DG. A decreased level of DG limits PC biosynthesis in cAMP-treated cells by decreasing the supply of DG for cholinephosphotransferase. The supply of CDP-choline can also limit PC biosynthesis. In cells treated with TPA increased levels of DG mediate translocation of CT to the ER where it is activated. Also shown are alternative pathways for the biosynthesis of PC from PE catalyzed by the PE methyltransferase (PEMT) or reacylation of lysophosphatidylcholine (lyso-PC) by an acylCoA acyltransferase (AT). The other abbreviations are: PKA, cAMP-dependent protein kinase; AdoMet, S-adenosylmethionine; AdoHcy, S-adenosylhomocysteine.

independent of PC hydrolysis but only because the signal that triggers PC biosynthesis is being provided without the necessity for hydrolysis. An increase in PC mass can be expected to occur due to DiC_8-stimulated PC biosynthesis. In fact no increase in cellular PC mass was observed (128 and 126 nmol/mg in control and DiC_8-treated cells, respectively). It is possible that PC does not accumulate because excess PC may be catabolized. Recently it has been shown that under certain conditions PC catabolism can be regulated in rat hepatocytes and that the concentration of PC might regulate its catabolic rate (Tijburg *et al.*, 1991). Furthermore, when PC biosynthesis was stimulated in Krebs II cells treated with oleic acid no accumulation of PC was observed, suggesting that PC biosynthesis and catabolism may be closely linked (Tercé, *et al.*, 1991).

4.2.5. *Unequivalent Effects of Different DG Analogues*

There is a detailed literature review by Kiss (1991) on the differences between effects mediated by PKC activation and those mediated by DG analogues observed by various investigators. This review, however, does not critically analyze many of the claims made in these reports. It is difficult to draw consistent conclusions from many of these reports as diverse cell types are used and there is a lack of consistency among investigators with respect to experimental design. In addition, the technical approaches to some of these experiments are questionable, for example, Hochberger *et al.* (1989) reported that suppression of Ca^{2+} currents in sensory neurons can be PKC-independent. One of the agents used in this study, a DG analogue (OaG) was reported by the authors as difficult to dissolve and found that it was effective only after storage in chloroform for 3-4 weeks at 4°C, a condition which makes the integrity of OaG questionable. Furthermore, these investigators used PKC

inhibitors such as 1-(5-isoquinolinylsulfonyl)-2-methylpiperazine (H-7), sphingosine and staurosporine of which H-7 and sphingosine in particular are non-specific agents.

Many of the non-specific or minor effects reported due to exogenous DGs may be merely artifactual or incomplete, for example, the method of addition of DiC_8 to cells may be important if maximum effects are to be achieved (Kolesnick and Hermer, 1990). However, DiC_8-induced increases in intracellular calcium appear to be unique to this DG analogue and independent of PKC (Ebanks *et al.*, 1989; Restrepo *et al.*, 1989), although as discussed in section 1.6 the different methods of detecting variations in intracellular Ca^{2+} may give inconsistent results.

In some instances conclusions drawn from studies in this area indicate more complex mechanisms than may be warranted, for example, Billah and Siegel (1987) conclude that phospholipase A_2 is activated because radiolabeled arachidonic acid release was augmented in cells that were prelabeled with [1-^{14}C]arachidonic acid and treated with DG analogues and other agents. Increased release of fatty acids does not necessarily indicate phospholipase A_2 activation. Since the levels of fatty acids were not measured the results could merely indicate increased turnover of PC due to increased PC biosynthesis.

Therefore, the literature in this area must be read with great caution and particular attention paid to experimental design and the conclusions drawn from the results. Furthermore, many of these studies need to be reevaluated.

4.2.6. *Incorporation of DiC_8 into PC*

It was shown in this study that DiC_8 was incorporated preferentially over long-chain DG into PC in PKC-downregulated HeLa cells treated with DiC_8. Fig. 40 shows that long-chain DG levels remained unchanged in DiC_8-treated cells

compared to controls. CPT does not exhibit substrate specificity with respect to the molecular species of DG as shown by Ide and Weinhold (1982) and incorporates at similar rates species of DG with different fatty acid compositions. In the present study it is possible that DiC_8 was more easily accessible to CPT as a substrate compared to long-chain DG, rather than due to a preference for DiC_8 as a substrate. The results also suggest that exogenous DG was transported to the site of CPT action, believed to be the endoplasmic reticulum (Cornell, R., 1989).

The mobility of DiC_8-PC on TLC plates using the solvent systems for phospholipid separation utilized in this study was different from that of long-chain PC. This enabled separation of the two species of PC. However, GLC analysis of silica gel scrapings containing DiC_8-PC suggests two things:

1) an unidentified lipid such as lysophosphatidylcholine comigrated with DiC_8-PC, or

2) the PC species analyzed did not contain solely the 8:0 fatty acid as would be expected for pure DiC_8-PC but remodelling occured by deacylation of one or both 8:0 chains and subsequent reacylation by other fatty acids. Indeed there was preliminary evidence (not shown) for this suggestion in which the mobility of DiC_8-PC was seen to shift over time towards that of long-chain PC. There is an important implication for this observation. It suggests that cells incorporate non-physiological or foreign fatty acids or DG's indiscriminiately, perhaps as a protectve mechanism from potential harmful effects, and that later remodeling occurs to provide the cells with more physiological species of lipids. The system in this study, therefore, may be potentially important as a model system in which to study remodelling and the enzymes involved in the process.

When the separation of DiC_8-PC from long-chain PC was done by a published procedure using a reversed phase C18 TLC system (Liscovitch *et al*, 1987), results similar to those in Figs. 44 and 45 were obtained (not shown). Therefore, claims of separation of DiC_8-PC from long-chain PC must be viewed with caution.

4.3. ELIMINATION OF OTHER POSSIBLE MECHANISMS OF TPA-ELICITED CT TRANSLOCATION

Several other potential mechanisms besides DG for CT translocation by TPA were investigated:

4.3.1. *Does Reversible Phosphorylation Play a Role in TPA-Elicited CT Translocation?*

Several lines of evidence indicated that the subcellular distribution of CT may be determined by its phosphorylation state. Pelech *et al.* (1981) observed an inhibition of PC biosynthesis in rat hepatocytes treated with stable analogues of cAMP. This was accompanied by an increase in cytosolic CT activity and a concomitant decrease in microsomal CT activity. It was proposed that under increased phosphorylation conditions CT would be hydrophillic due to increased phosphate groups and, therefore, unable to associate with membranes. *In vitro* evidence in support of this mechanism was provided by Sanghera and Vance (1989) who showed that purified CT could be phosphorylated by cAMP-dependent protein kinase and that dephosphorylation of the purified enzyme with alkaline phosphatase caused it to associate with membranes. More recently, Hatch *et al.* (1991) have demonstrated an inhibition of PC biosynthesis and a redistribution of microsomal CT to the cytosol in rat hepatocytes treated with okadaic acid, a

specific inhibitor of protein phosphatases 1 and 2A. There is evidence that the phosphorylation state of CT increases in the presence of okadaic acid (Hatch *et al.*, submitted for publication) further corroborating the reversible phosphorylation hypothesis. In another system, Thelen *et al.* (1991) have shown that the phosphorylation state of a myristoylated PKC-substrate can reversibly modulate its association with the plasma membrane.

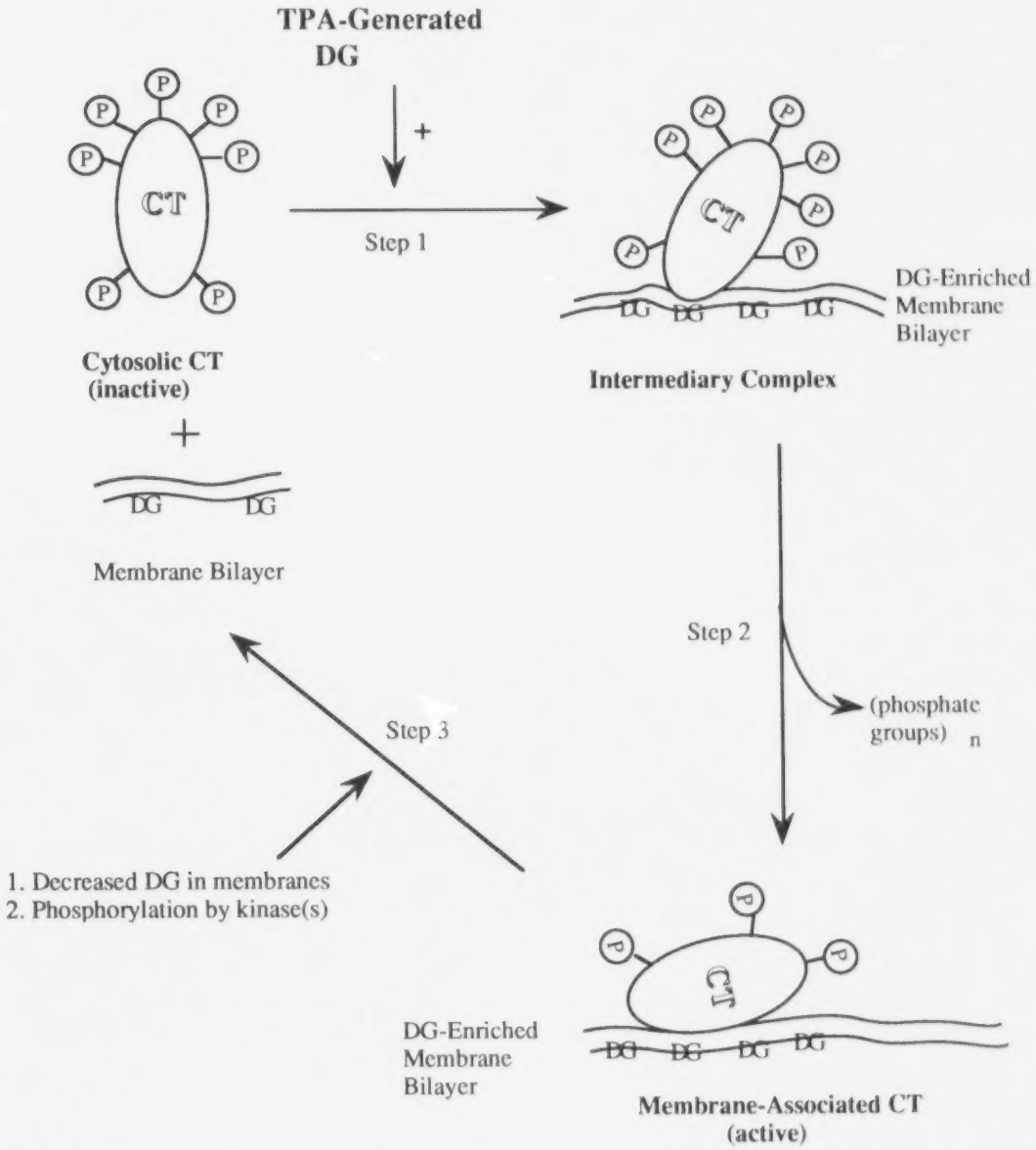
However, the translocation of CT to membranes in TPA-treated HeLa cells in the present study did not appear to be mediated by a phosphorylation /dephosphorylation mechanism. Immunoprecipitation of CT from controls and TPA-treated cells did not reveal any obvious changes in the phosphorylation state of cytosolic CT (Figs. 49 and 50), although it appears to be a highly phosphorylated protein. This latter observation agrees with that of Watkins and Kent (1990). Indeed, an analysis of the amino acid sequence deduced from the cDNA sequence of rat liver CT reveals several potential sites for CT to be phosphorylated (Kalmar *et al.*, 1990). It is possible that there are subtle changes in the phosphorylation state of CT which are not obvious in the present study.

Densitometer scanning of the autoradiograph obtained after immunoprecipitation of membrane-associated CT from TPA-treated cells showed a 50% more intense band compared to that from controls (Fig. 49). This increase can be attributed to more enzyme present on the membrane after TPA treatment. However, if this were the case, then a three-fold more intense band would be expected from TPA-treated cells as compared to controls. Since this is clearly not the case as seen in Fig. 49, several explanations can be offered. In a highly speculative vein it is possible that a membrane-associated phosphatase already present or activated by DG may dephosphorylate CT after it has translocated to membranes according to a proposed model illustrated in

Fig. 59. Indeed microsomal phosphatases have been reported (Alemany *et al.*, 1986; Mieskes and Söling, 1987) although the literature on the subcellular distribution of phosphatases is sparce. More recently, Alexander *et al.* (1988) have demonstrated by immunoblotting techniques the association of type 1, 2A and 2B protein phosphatases with the plasma membrane of human T lymphocytes, whereas previously Cohen (1985) had reported type 1 as being membrane-associated and types 2A and 2C as being entirely cytosolic. These inconsistencies appear to be due to the different substrates used to assay for the presence of phosphatases and the varied fractionation techniques employed by different investigators. Not all phosphatases dephosphorylate a standard substrate used in assays equally, for example, Takai and Mieskes (1991) have shown that of the types 1, 2A and 2C protein phosphatases, 2A has the highest activity towards *p*-nitrophenylphosphate whereas 2C exhibits weak activity towards this substrate. Gschwent *et al.* (1984) have reported TPA-stimulated alkaline phosphatase activity in mouse epidermis. The significance of this result is unclear as the authors reported cytosolic activity whereas alkaline phosphatase including all its isozyme forms in HeLa S3 cells have been reported to be principally localized at the plasma membrane (Tokumitsu, 1984). Therefore, there appears to be a need for investigating specific naturally-occuring substrates of different phosphatases and their subcellular distribution before relevant conclusions about stimulation or inhibition of phosphatases can be drawn.

Another explanation for the results of the immunoprecipitation studies on membrane-associated CT is that a soluble phosphatase activated by PKC or DG may dephosphorylate CT and cause it to translocate to membranes. This possibility appears to be unlikely for two reasons:

Fig. 59. Proposed Model for a Role for a Phosphatase in CT Association with Membranes. Increased DG due to TPA-treatment signals the translocation of cytosolic CT with its full complement of phosphate groups to membranes, either by directly binding to CT or by facilitating the binding of CT by an effect on membrane structure (Step 1). A phosphatase present on the membranes removes several phosphate groups from the enzyme, thereby enhancing its binding to the membrane bilayer (Step 2). For a reversal of this translocation the enzyme dissociates from the membrane upon depletion of DG, and upon reverting to the cytosol is phosphorylated by cytosolic kinase(s) (Step 3).


TPA-Generated
DG
+
Step 1
CT
P
DG-Enriched
Membrane
Bilayer
DG
Cytosolic CT
(inactive)
Intermediary Complex
+
Membrane Bilayer
Step 2
(phosphate
groups) n
Step 3
1. Decreased DG in membranes
2. Phosphorylation by kinase(s)
DG-Enriched
Membrane
Bilayer
Membrane-Associated CT
(active)

1) the phosphorylation state of *cytosolic* CT appears to be unchanged by TPA-treatment although more subtle effects cannot be discounted at the present time. This can be resolved by comparing peptide maps of immunoprecipitated ^{32}P-labeled cytosolic CT from control and TPA-treated cells.
2) a continued dephosphorylation by an activated soluble phosphatase would result in more translocation of CT to membranes than is seen in the present study.

Therefore, it is highly likely that dephosphorylation may be a secondary event to the initial translocation of CT to membranes and could reinforce the association of CT with membranes by making CT more hydrophobic.

Experiments with okadaic acid in the present study did not inhibit TPA-stimulated CT translocation in HeLa cells. Since okadaic acid has been shown to inhibit only protein phosphatases types 1 and 2A and polycation modulated phosphatase (PCM) (Bialojan and Takai, 1988; Takai and Mieskes, 1991) some other phosphatase may dephosphorylate CT. Indeed, a number of phosphatases have been reported to be insensitive to okadaic acid (Bialojan and Takai, 1988; Takai and Mieskes, 1991).

CT appears to be a highly phosphorylated protein as seen in the present study and as reported by Watkins and Kent (1990). The significance of this, if any, remains to be established. A high degree of phosphorylation may be required to elicit structural changes that affect enzyme function. Alternatively, the phosphorylation state of CT may have nothing to do with its function. Since cytosolic CT is believed to serve as a reservoir one possible reason for its highly phosphorylated state may be to prevent its degradation. A high negative surface charge on CT might be incompatible with protease binding and prevent proteolytic enzymes from gaining access to potential degradation sites. Consistent with this latter hypothesis is evidence that rat

liver CT has a relatively long half-life of approximately 21 h (Jamil, H. and Vance, D.E., unpublished observations).

4.3.2. *Intracellular PC levels*

Previous studies from this laboratory have established a role for PC levels as a negative feedback regulation of its synthesis; PC levels in rat liver Yao *et al.*, 1990) and rat hepatocytes (Jamil *et al.*, 1990) were shown to modulate the association of CT with membranes. Since the DG accumulation upon TPA-treatment is the result of PC hydrolysis (Glatz *et al.*, 1987) it was conceivable that the TPA-elicited CT translocation observed in the present study was due to decreased PC levels. PC levels remained unchanged in both controls and TPA-treated cells (section 3.4.3). Moreover, the experimental approach used in which PKC-downregulated cells were treated with DiC_8 is indicative of increased DG, rather than decreased PC, causing CT translocation to membranes.

An additional discovery made in the time-course study of DiC_8-elicited CT translocation (Fig. 37) was that DiC_8 caused maximal CT translocation to membranes by 15 min, which then resumed to near normal levels by 1 h. The significance of this finding is unknown at the present time. The decrease in membrane-associated CT activity after 1 h of DiC_8 treatment could be due to either reduction in intracellular DiC_8 levels due to its rapid metabolism, or an increase in PC levels at the site of synthesis. PC concentrations have previously been shown to alter the amount of membrane-associated CT (Yao *et al.*, 1990; Jamil *et al.*, 1990). Although no increase in cellular PC was found upon DiC_8 treatment (128 and 126 nmol/mg in control and DiC_8-treated cells respectively after 1 h), this mechanism cannot be unequivocally eliminated at the present time. Localized intracellular sites may exist where fluctuations in

PC content may assume significance. It is also possible that excess PC may be catabolized. Recently, it has been shown that under certain conditions, PC catabolism can be regulated in rat hepatocytes (Tijburg *et al.*, 1991). Furthermore, when PC biosynthesis was stimulated in Krebs-II cells treated with oleic acid no accumulation of PC was observed, suggesting that PC synthesis and catabolism may be closely linked (Tercé *et al.*, 1991).

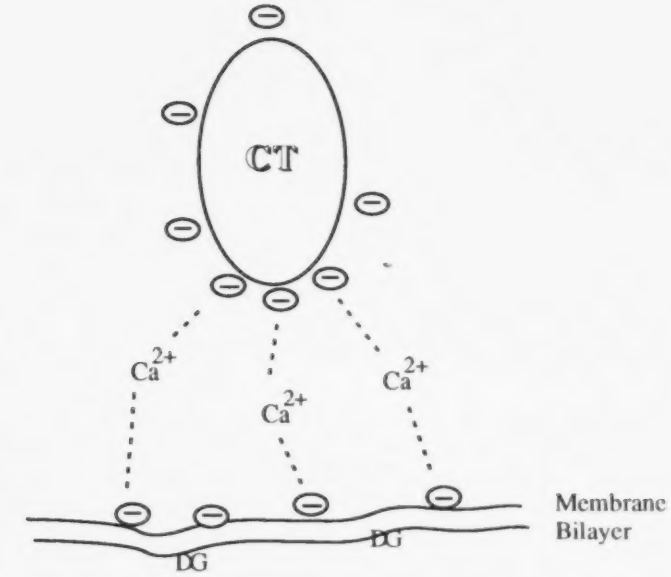
4.4. ARE THE EFFECTS OF CYTOCHALASIN E ON TPA-STIMULATED PC BIOSYNTHESIS MEDIATED BY Ca^{2+}?

Studies with cytochalasin E, a microfilament-disrupting agent, were undertaken to investigate any association of actin with CT. Since TPA and DiC_8 have been reported to cause the polymerization of actin (Rao, 1985; Keller, 1985), it was possible that the CT translocation elicited by TPA and DiC_8 were mediated by an association of CT with actin. In addition, Hunt *et al.* (1990) suggested that CT in human and rat lung associated with actin although the evidence was largely circumstantial. Surprisingly, instead of inhibiting TPA-stimulated PC biosynthesis cytochalasin E was found to enhance significantly the incorporation of [^{3}H]choline into PC in the presence of TPA (section 3.5.2). This was possibly due to an increase in intracellular calcium since cytochalasin E is known to mobilize intracellular calcium (Treves *et al.*, 1986). Since many phospholipases such as phospholipase A, C and D require calcium for activity (Waite, 1987), an increased hydrolysis of PC over that in TPA-treated Hela cells could result in increased synthesis of PC. A measurement of DG levels, however, indicated that phospholipase C activity was not enhanced. In fact, DG levels in cells incubated in the presence of both TPA and cytochalasin E were observed to *decrease* compared to cells that were treated with TPA alone (Fig. 54) indicating that the increased PC biosynthesis had

decreased the pool of DG due to its utilization by cholinephosphotransferase. An alternative explanation for the enhanced PC biosynthesis could be an increased PKC activation since this enzyme is Ca^{2+}-dependent. However, the decreased DG levels in the presence of TPA and cytochalasin E argue against this. The results indicated an enhancement of CT activity and this was indeed found to be the case (Table 6). It was proposed that calcium directly modulated CT association with membranes. In light of the results obtained it is now proposed that calcium causes a loose and non-functional association of CT with membranes; loose because inclusion of 5 mM EGTA in the digitonin-buffer returned membrane CT in cytochalasin E-treated cells to control levels; non-functional because, despite the increased membrane-associated CT in cytochalasin E-treated cells, PC biosynthesis remained the same as that in controls.

Further elaborating, it is proposed that a loose association of CT with membranes 'primes' DG-mediated CT translocation. In such a model (schematically presented in Fig. 60) Ca^{2+} molecules would form 'bridges' between the negatively charged phosphate groups of CT and the acidic phospholipids of membranes thereby placing CT in a strategic position for intercalation with membranes. The presence of DG would facilitate hydrophobic and functional association of CT with membranes and Ca^{2+} chelators such as EGTA would then be unable to dissociate CT from the membranes. In the presence of cytochalasin E alone, membrane-associated CT could be dissociated from membranes by EGTA, since insertion into membranes would not take place without the production of DG. This model is an extrapolation of that proposed for interaction of PKC with membranes (Bazzi and Nelsestuen, 1989).

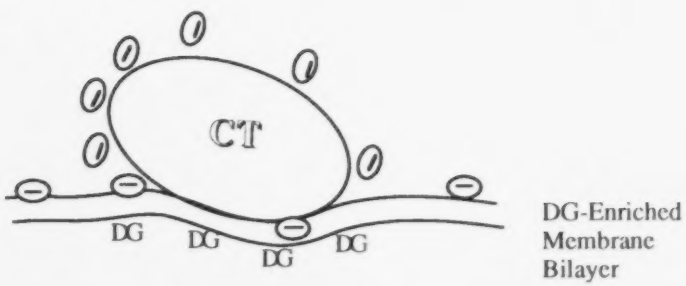
Fig. 60. Proposed Model for Ca^{2+}-Mediated CT Translocation to Membranes. Ca^{2+} molecules form 'bridges' between the negative charges on CT (possibly the phosphate groups) and anionic phospholipids such as phosphatidylserine in the membrane bilayer (CT is highly activated by negatively charged phospholipids, Cornell, R., 1991a). This serves to bring the enzyme closer to the membrane bilayer where the binding of CT is facilitated by an increase in DG due to TPA-treatment.



Membrane-Associated CT
(Inactive)

TPA-
Generated
DG

+



Membrane-Associated CT
(Active)

4.5. THE SUBCELLULAR DISTRIBUTION OF CT AT DIFFERENT STAGES OF CELL GROWTH IS UNCHANGED

Studies on the subcellular localization of CT using immunocytochemical methods led to an investigation of the subcellular distribution of CT activity in cells at different stages of growth (section 3.6.3). It was expected that when the cells were in an active log phase, a large percentage of the total CT activity would be membrane-associated. Surprisingly, there was no change in subcellular distribution of CT in actively growing cells compared to confluent cells or cells made quiescent by serum-deprivation (Table 7). Neither was there a change in the total amount of CT activity in these cells. This suggested that a cell went through life with an unchanging complement of CT whose subcellular distribution was regulated by external stimuli rather than the phase of growth the cell was in.

4.6. TRANSFECTION OF HELA CELLS WITH YEAST PHOSPHOLIPID METHYLTRANSFERASE

In eukaryotic cells PC can also be synthesized by methylation of PE. In rat liver 40% of PC is synthesized by three successive methylations of PE by one enzyme, phosphatidylethanolamine methyltransferase (Ridgeway, 1989). In HeLa cells there is no evidence that the PE methylation pathway is operative and preliminary assays of methyltransferase activity by Pelech and Vance (unpublished observations) in HeLa cell extracts were devoid of any detectable activity.

The entire coding sequence of the yeast enzyme has been cloned by Kodaki and Yamashita (1987). In order to study the enzyme further HeLa cells were transfected with a plasmid containing the phospholipid methyltransferase

gene (*PEM2*) and selected for gentamycin sulfate (G418) resistance as described in section 2.24 and as illustrated in Fig. 61. The second aim of introducing the methyltransferase gene into HeLa cells was to study the resulting effect, if any, on the CDP-choline pathway.

Before any studies on the effect of the methyltransferase on PC and PE metabolism were conducted an attempt was made to see if this enzyme could rescue HeLa cells from possible deleterious effects of choline-depletion. These studies were inconclusive because choline-depletion did not result in the death of HeLa cells and no difference in growth was observed between control and transfected cells, although choline has been described as an essential vitamin for this cell-line by Eagle (1955). In both control and transfected cells growth was slow when put in choline-deficient medium. There are four possible reasons for the lack of an effect of choline-depletion:

1) The cells did not become choline-deficient perhaps because HeLa cells may efficiently recycle choline-containing metabolites. Indeed, Esko and Matsuoka (1985) have suggested that the choline derived from PC hydrolysis may be recycled into PC in choline-depleted CHO cells.

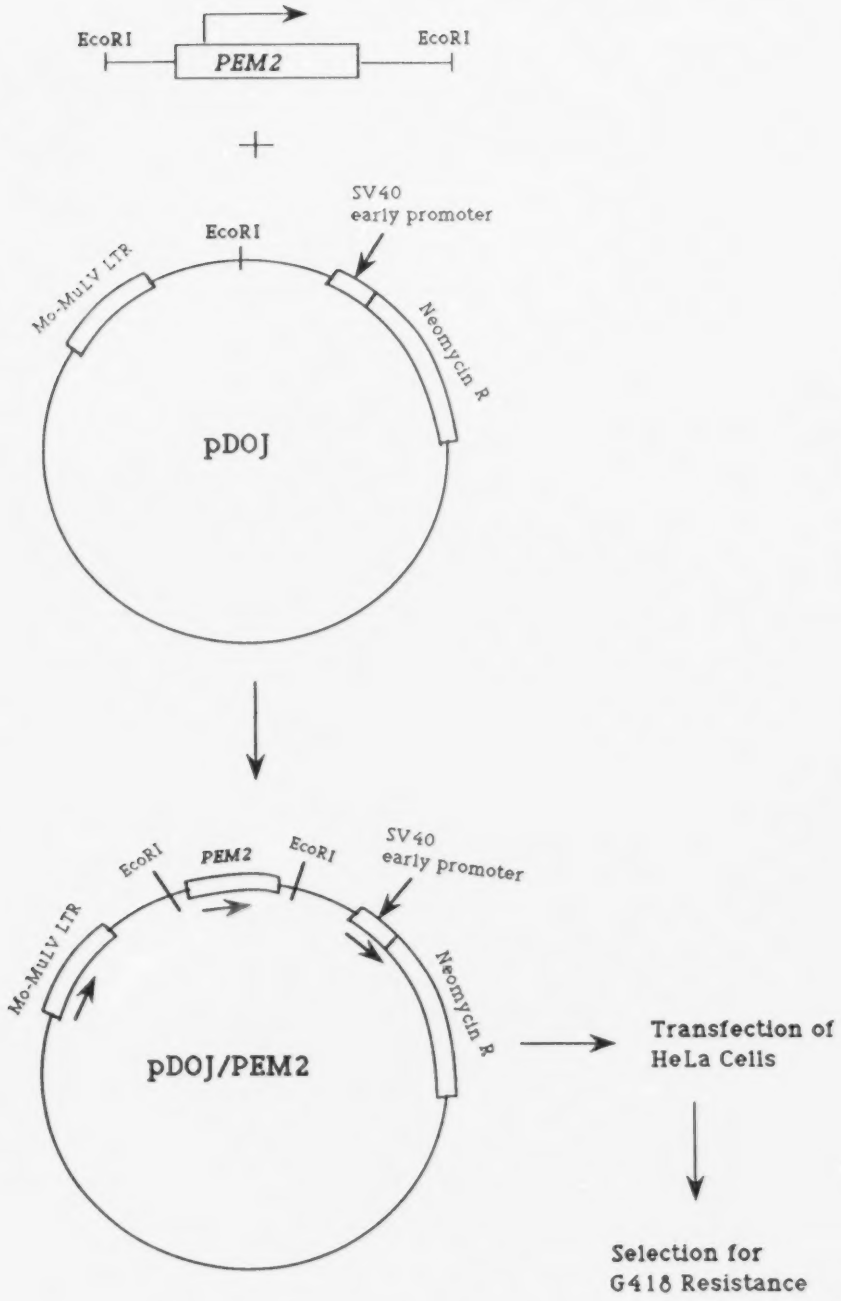
2) Some PEMT activity may have been induced in control cells in response to choline deficiency as an alternate means of supplying PC.

3) Since the growth of transfected cells was similar to that of control cells in choline-deficient medium, it was possible that the transfected yeast gene was not being functionally or otherwise expressed.

4) Yeast PEM2 catalyzes the first methylation step i.e., conversion of phosphatidylethanolamine to phosphatidylmonomethylethanolamine (PMME) slowly (Kodaki and Yamashita, 1987). It was possible that incubating the cells in the presence of monomethylethanolamine and therefore, bypassing the

first methylation so that it was no longer rate-limiting, would have accelerated the growth of the transfected cells over that of controls.

Fig. 61. Construction, Transfection and Selection of an Expression of an Expression Vector for *PEM2*. This figure is a schematic outline (not drawn to scale) of the construction of an expression vector for the yeast *PEM2* gene, its transfection into HeLa cells and its subsequent selection for G418 resistance. The abbreviations are, Mo-MuLV LTR (mouse-murine leukemia virus long terminal repeat); SV 40 (Simian Virus 40).


EcoRI
PEM2
EcoRI
SV40
early promoter
EcoRI
Mo-MuLV LTR
Neomycin R
pDOJ
EcoRI
PEM2
EcoRI
SV40
early promoter
Mo-MuLV LTR
Neomycin R
pDOJ/PEM2
Transfection of
HeLa Cells
Selection for
G418 Resistance

CHAPTER 5 CONCLUSIONS AND FUTURE DIRECTIONS

The main objective of this work was to elucidate the mechanism of TPA-elicited translocation of CT to membranes in Hela cells. Studies on the mechanism of translocation of this enzyme in the initial stages of this work were hampered to a large extent by the unavailability of a precipitating antibody, although the enzyme had been purified to homogeniety some years earlier (Weinhold *et al.*, 1986; Feldman and Weinhold, 1987). Success was achieved by Dr. Haris Jamil who was able to raise rabbit antibodies to a 13 amino acid sequence (amino acid residues 164 to 176) deduced from the nucleotide sequence of cloned rat liver CT (Kalmar *et al.*, 1990). Using this antibody it was possible to show by immunoblot analysis that translocation of the enzyme protein to membranes occurred upon TPA-treatment of HeLa cells.

The lack of a reversible phosphorylation mechanism in TPA-elicited CT translocation was demonstrated by immunoprecipitation of CT from ^{32}P-labeled HeLa cells treated with TPA. However, there were indications that a dephosphorylation of CT may be involved as a secondary event to its initial translocation to membranes. There was a lack of evidence supporting the involvement of membrane PC levels in modulating binding of CT to membranes. In addition, there was lack of evidence supporting aggregation or covalent lipid modification as mechanisms whereby TPA might cause CT to translocate to membranes.

Increased levels of DG were shown to cause CT translocation due to TPA-treatment. This was supported by increasing intracellular levels of DG by avoiding PC hydrolysis and incubating cells with an exogenous source of DG, DiC_8. DiC_8 was found to be incorporated preferentially over long chain DG.

A lack of involvement of the cytoskeleton in TPA-stimulated PC biosynthesis was demonstrated by the use of cytoskeletal-disrupting agents. Similarly, a lack of involvement of actin was also demonstrated. Serendipitiously, the studies with cytochalasin E enhanced TPA-stimulated PC biosynthesis via increased CT association with membranes. A direct interaction of Ca^{2+} with CT was indicated by preliminary studies.

With the availability of an immunoprecipitating antibody and cloned rat liver CT, elucidation of enzyme structure and regulation can now begin in earnest. There is abundant literature on lipid and other activators of CT. The most advances in the study of this enzyme are likely to come from the utilization of molecular biology combined with immunological techniques. The following approaches appear to be promising:

1) Deletion mutants of cytosolic CT can be used to study the peptide domains of CT that may be important in binding to membranes. Indeed this work is in progress in the laboratory of Dr. Rosemary Cornell at Simon Fraser University, Vancouver. Preliminary results indicate that the COOH-terminal domain of CT may be important in its interaction with DG (personal communication).

2) To confirm and understand a potential role of a reversible phosphorylation mechanism in subcellular distribution of CT, peptide map analyses of immunoprecipitated CT from ^{32}P-labeled control and TPA-treated cells are important. A comparison of the peptide maps of cytosolic and membrane-associated CT is imperative to study a dephosphorylation event in CT translocation.

3) As an extrapolation of a reversible phosphorylation mechanism, studies can be performed to find out whether the translocated membrane-associated CT is made more hydrophobic by virtue of dephosphorylation. This can be

done by extracting CT from whole cells or subcellular fractions using Triton X-114. This non-ionic detergent is homogeneous at 0°C and separates into aqueous and detergent phases above 20°C and can be used to separate hydrophillic proteins from membrane proteins (Bordier, 1981).

4) The importance of studying the interaction between different signal transduction pathways is apparent in the literature. This is particularly true in the regulation of phospholipid metabolism. Work from this laboratory has shown that DG occupies a central position in the regulation of PC biosynthesis (Utal *et al.*, 1991; Jamil *et al.*, 1992). A logical extrapolation of these studies would be to investigate any interaction between the cAMP-mediated inhibition of PC biosynthesis and TPA-stimulated PC biosynthesis and CT translocation. Would the pathways activated by these two agents interact negatively with each other, or are they independent of each other?

5) There is evidence that PE-derived PC is more susceptible to TPA-stimulated hydrolysis in NIH 3T3 cells than is PC derived from from the CDP-choline pathway (Kiss, 1990). I calculated a 77% release after an unspecified time of [^{14}C]choline into the medium of TPA-treated cells prelabeled with [^{14}C]ethanolamine compared to a 4.2% release of [^{14}C]choline from cells similarly treated and prelabeled. In addition, TPA appeared to enhance the formation of PC through the methylation pathway. This is an exciting observation. It suggests that HeLa cells transfected with a rat liver cDNA clone of CT (Dr. Zheng Cui in this laboratory is currently on the verge of isolating a full length rat liver cDNA) will provide a powerful model in which to study the coordinate regulation of the two pathways of PC biosynthesis, namely the CDP-choline and PE methylation pathways. It can also be used to study the mechanism whereby TPA may modulate PC biosynthesis via the latter pathway. An antibody can be generated against PEMT and the effects of TPA, if any, on

the phosphorylation state of PEMT can be studied to find out whether this enzyme is regulated by a phosphorylation mechanism.

6) Remodelling of PE species in rat hepatocytes has been studied by R. Samborski in this laboratory. There is preliminary evidence in the present study that DiC_8-PC may be remodelled to a PC species with a more physiological composition of acyl chains. Therefore, DiC_8-treated PKC-downregulated cells appear to be a potentially good model to study remodeling of phospholipid species and the enzymes involved in the process.

7) A long term project would be to identify and purify the PC-specific phospholipase C or D involved in the hydrolysis of PC in response to TPA-stimulation.

Finally, a philosophical question remains - is the large cytosolic pool of CT merely a reservoir or does CT have another as yet unknown function?

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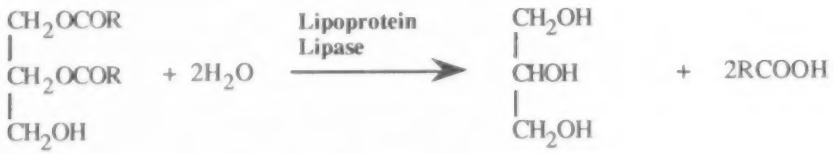
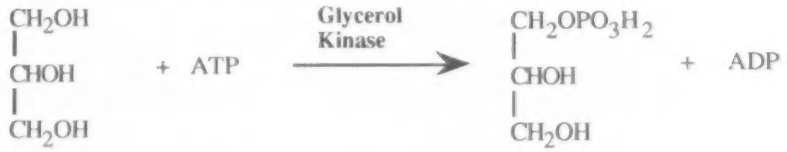
APPENDIX A

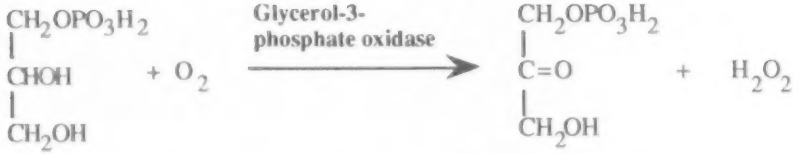
The principle underlying the method for measuring DG using the triglyceride assay kit is outlined as follows:



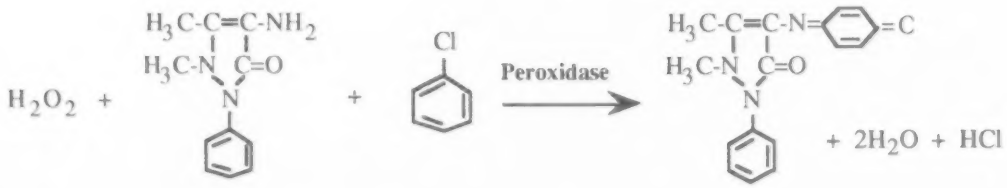
Diglyceride glycerol



Glycerol - 3 - phosphate



Dihydroxyacetone phosphate

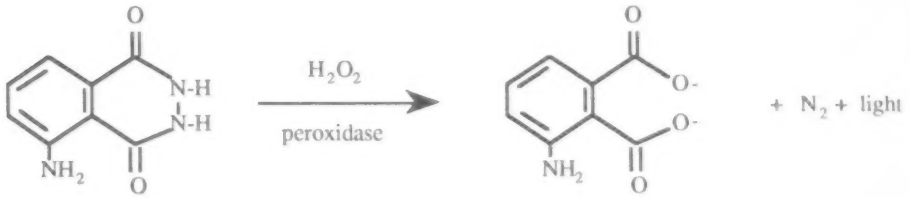


4 - Amino-antipyrine *p*-chlorophenol

APPENDIX B

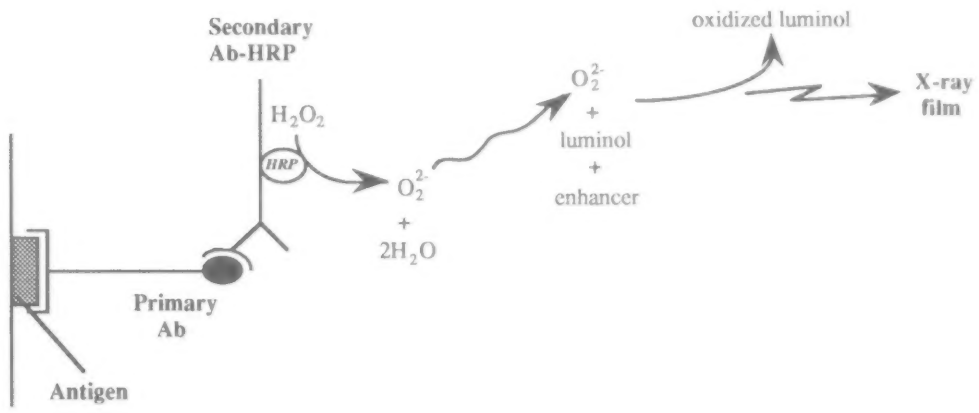
Antigen detection using enhanced chemiluminescence (ECL)

This method was used because of its high sensitivity. The principle of the method is described briefly. A secondary antibody that is conjugated to horseradish peroxidase (HRP) is used after incubation with the primary antibody. The HRP catalyses the oxidation of luminol in the presence of hydrogen peroxide (H_2O_2) as shown below.

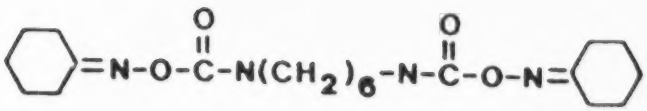


luminol 3 - aminophthalate

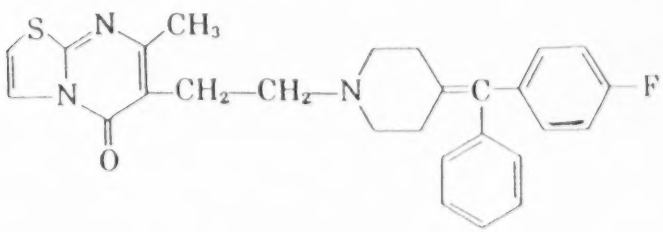
The oxidized luminol is in an excited state which decays to the ground state via a light emitting pathway. The emitted light is enhanced by certain phenol derivatives and detected by exposure to X-ray film. The overall scheme is illustrated as follows:



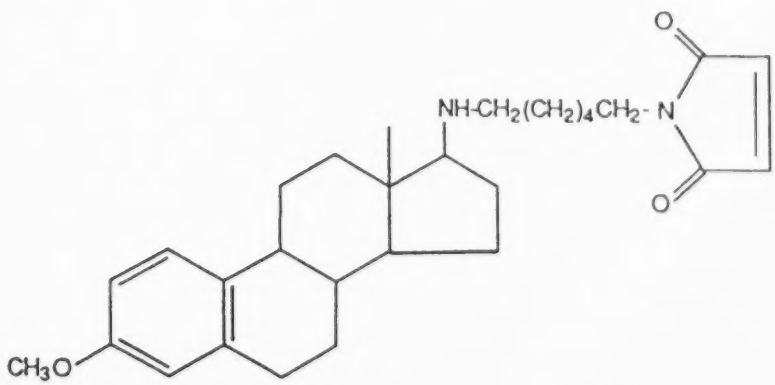
APPENDIX C



Diacylglycerol Lipase Inhibitor (U-57908, formerly RHC 80267)
1,6-di(O-(carbamoyl)cyclohexanone oxime)hexane
(from Sutherland and Amin, 1982)



Diacylglycerol Kinase Inhibitor (R-59022)
6-[2-(4-[(4-fluorophenyl)phenylmethylene]-1-piperidinyl]ethyl]-7-methyl-5*H*-thiazolo[3,2-α]pyrimidine-5-one
(from de Chaffoy de Courcelles *et al.*, 1985)



Phospholipase C Inhibitor (U-73122)
1-[6-[[17β-3-Methoxyestra-1,3,5(10)-Trien-17-yl]Amino]Hexyl]-1H-Pyrrole-2,5-Dione
(from Bleasdale *et al.*, 1990)